

Marilanda Ferreira Bellini

**Estudos imuno-histoquímico das proteínas p53, p16,
Fhit, caspase 3 e antígeno Ki67; e citogenético
molecular em lesões benignas e carcinoma de
esôfago**



UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de São José do Rio Preto

Programa de Pós-Graduação em Genética

Marilanda Ferreira Bellini

Estudos imuno-histoquímico das proteínas p53, p16, Fhit, caspase 3 e antígeno Ki67; e citogenético molecular em lesões benignas e carcinoma de esôfago

Orientadora: Profa. Dra. Ana Elizabete Silva

Co-Orientadora: Profa. Dra. Marileila Varella-Garcia

**Tese apresentada para
obtenção do Título de Doutor
em Genética**

**São José do Rio Preto – SP
2009**

Bellini, Marilanda Ferreira.

Estudos imuno-histoquímico das proteínas p53, p16, Fhit, caspase 3 e antígeno Ki67; e citogenético molecular em lesões benignas e carcinoma de esôfago/ Marilanda Ferreira Bellini. - São José do Rio Preto : [s.n.], 2009.

219 f. : il. ; 30 cm.

Orientador: Ana Elizabete Silva

Co-orientador : Marileila Varella-Garcia

Tese (doutorado) – Universidade Estadual Paulista, Instituto de

Biociências, Letras e Ciências Exatas

1. Genética. 2. Chagas, Doença de. 3. Megaesôfago chagásico. 4. Esofagite crônica. 5. Carcinoma de células escamosas. 6. Esôfago - Doenças. 7. Lesões esofágicas benignas. I. Silva, Ana Elizabete. II. Varella-Garcia, Marileila. III. Universidade Estadual Paulista, Instituto de Biociências, Letras e Ciências Exatas. IV. Título.

CDU - 575

Marilanda Ferreira Bellini

Estudos imuno-histoquímico das proteínas p53, p16, Fhit, caspase 3 e antígeno Ki67; e citogenético molecular em lesões benignas e carcinoma de esôfago

Dissertação apresentada para obtenção do título de Doutor em Genética, junto ao Programa de Pós-Graduação em Genética do Instituto de Biociências, Letras e Ciências Exatas, da Universidade Estadual Paulista “Júlio de Mesquita Filho”, Campus de São José do Rio Preto.

BANCA EXAMINADORA

Profa. Dra. Ana Elizabete Silva
Professor Adjunto Livre-Docente
UNESP – São José do Rio Preto
Orientadora

Profa. Dra. Juliana Karina Ruiz Heinrich
Bióloga
Universidade Estadual de Campinas

Profa. Dra. Agnes Cristina Fett Conte
Professor Livre-Docente
Faculdade de Medicina de São José do Rio Preto

Profa. Dra. Silvia Regina Rogatto
Professor Adjunto Livre-Docente
UNESP – Botucatu

Profa. Dra. Paula Rahal
Professor Adjunto Livre-Docente
UNESP – São José do Rio Preto

São José do Rio Preto, 20/02/2009.

O presente trabalho foi realizado no Laboratório de Citogenética e Biologia Molecular Humana, Departamento de Biologia do Instituto de Biociências, Letras e Ciências Exatas de São José do Rio Preto – SP, da Universidade Estadual Paulista “Júlio de Mesquita Filho” – UNESP; Laboratório de Imuno-histoquímica e Colorações Especiais, Seção de Patologia do Hospital de Base de São José do Rio Preto – SP; Laboratório de Patologia Cirúrgica e Molecular, Hospital Sírio Libanês, São Paulo – SP e Cytocore, University of Colorado, Health Sciences Center, Aurora – CO – USA, com bolsa de Doutorado do CNPq, bolsa de estágio de doutoramento no exterior CAPES e auxílio financeiro da FAPESP.

Dedicatória

Aos meus pais, Pedro Edgard Bellini e Cleonice Maria Ferreira Bellini, e a minha “irmãzinha” Franciele Ferreira Bellini, pois não deve ter sido nada fácil me apoiar minhas peripécias e idéias de cientista lunática.

Agradecimentos

“Senhor,

Dai-me serenidade para aceitar as coisas que não posso
mudar;

Coragem para mudar as que eu posso e

Sabedoria para distinguir umas das outras.”(E. K. Ross,D.Kessler)

A Deus, “por ordenar o mundo de maneira
tão bela e sábia”, permitindo que sempre se abra uma janela, quando todas
as portas parecem estar fechadas;

“Eu tenho tanto pra te falar,

mas com palavras não sei dizer, como é grande o meu amor por você,

Nem mesmo o céu, nem as estrelas, mesmo o sol ou o infinito, não
conseguem ser maior que o meu amor, nem mais bonito” (Vinicius de
Moraes)

A minha Família pelo AMOR incondicional;

“Imagine how the world could be

So very fine

So happy together” (Garry Bonner e Alan Gordon)

A Juliano Farias da Nóbrega pela parceria,
companheirismo, apoio e incentivo.

“Mestre, posso sair mais tarde hoje?

Ao contrário do que algumas vezes te pedi, hoje quero ficar mais um pouco.

Ficar mais alguns instantes para desfrutar de tua presença e da proteção
que ela me traz.

Ficar, para poder admirar, mais uma vez, a tua destreza, a tua capacidade
de diagnosticar e prognosticar. Ficar pela segurança, pelo poder contar,
contar contigo, amparando minhas dúvidas e, suscitando em mim a
maturidade de um profissional...

Agora, encontro-me, procurando meu estado de aluno, sabendo que em instantes me tornarei seu colega. Porém, com a certeza de jamais deixar de ser seu discípulo. Discípulo que guardará na memória seus ensinamentos e no coração, a gratidão, o respeito e a saudade” (*Anônimo*)

As **Profª Drª Ana Elizabete Silva** e **Profa. Dra. Marileila Varella-Garcia** pela confiança, ensinamentos e amizade;

A Banca Examinadora, **Profa. Dra. Silvia Regina Rogatto**, **Profa. Dra. Juliana K.R. Heinrich**, **Profa. Dra. Agnes C. Fett-Conte** e **Profa. Dra. Paula Rahal** por terem aceitado a participar da análise e enriquecimento do trabalho.

Aos meus amigos que “como anjos me levantaram, quando minhas asas tiveram problemas, me lembrando como voltar a voar” (*Mário Quintana*);

“O mais altruísta dos amigos que um homem pode ter neste mundo egoísta, aquele que nunca o abandona e nunca mostra ingratidão ou deslealdade, é o cão”. (*Anônimo*)

A todos animais de estimação, que passaram pelo meu percurso, pelos rabinhos abanando de felicidade, e em especial a **Bella**, pela felicidade ao me reencontrar depois de um ano e pelo companheirismo confidente, meses e meses, sempre ao meu lado, durante a confecção desta Tese;

“Se tem bigodes de foca, nariz de tamanduá;
Parece meio estranho, heim?
Também um bico de pato e um jeitão de sabiá;

Mas é amigo, não precisa mudar, é tão lindo deixa assim como está”
(*Roberto Carlos*)

Aline Poersch, Andressa Seri, Arianca Alvarado, Cíntia Nunes, Claudiane Borba, Edson Lopes, Gislaine Rodrigues, Mateus Marcos, Milene Lara e Patrícia Batista, por não se importarem com as diferenças, desavenças e distância;

Isabeth Estevão, Ligia Baracioli, Lucilene Maschio e Rita Peruquetti, a esquerda festiva, por também “esquecerem o que falaram sobre ser adulto” (*Arnaldo Jabor*) e terem dividido suas idiotices comigo;

Cadu Barbosa, Fernanda Carregaro, Franciole e Tatiana Caparroz, Gilmar Santos, Janaina Rigonatto, Leonardo Cabrioti, Marco “Todd” Asanome, Marta Lovato, Mauro Leonardo, Nayara Delgado, Patrícia Ayub, Rodrigo Juliano, Ronaldo Costa e Tázio Gaspari, “por me guardarem do lado esquerdo do peito, mesmo que tempo e a distância digam não” (*Milton Nascimento*).

Luciana Ondeí, Paula Zamaro, Ana Carolina, Ana Luiza, Profa. Dra. Claudia Regina Bonini Domingos, Nelson C. Tukamoto Jr, Carlos Zago, por se serem AMIGOS até nos momentos mais difíceis.

*“Don’t stop believin
Hold on to the feelin
Streetlight people” (James Iha)*

Diana Villa, Estefânia Muriedas e Elvia Navarro, por serem as melhores *muchachas* em terras tão distantes.

Aos meus primos **Monique, Wanderson, Ivan, Bruna, Ligia, Juliano e Michele** por me aceitaram como sou e se divertirem com isso, sempre oferecendo o ombro amigo.

“Pane no sistema, alguém me desconfigurou” (*Pitty*)

A **Flayd Guerreiro e Juliano Farias Nóbrega** por toda assessoria excell, photoshop, corel draw e afins;

*“When you’re down and troubled
And you need some loving care
And nothing, nothing is going right
Close your eyes and think of me and soon
I will be there to brighten up even your darkest night*

...

Ain’t it good to know you’ve got a friend...” (*Carole King*)

Marcelo Almeida, Milena Andrade, Juliano Farias, Manuela Santoro, Rodriguinho, Bruninha, “The Class Team”, pela diversão durante o prepatório para TOEFL Test *and friendship*.

“O valor das coisas não está no tempo que elas duram, mas na intensidade com que acontecem. Por isso, existem momentos inesquecíveis, coisas inexplicáveis e pessoas incomparáveis”. (*Fernando Pessoa*)

Aos colegas do **Laboratório de Citogenética e Biologia Molecular Humana**, **Cidinha Silveira**, **Daniela Bizari**, **Fernanda Manoel-Caetano**, **Héctor Matioli**, **Isabella Migliori**, **Josué Rodrigues**, **Juliana Oliveira**, **Márcia Duarte** e **Yvana Jorge**.

Ao **Pessoal do Setor de Patologia do Hospital de Base**, **Dinha** por repassar meus telefonemas constantes, **Andréia** por estar sempre disponível em me auxiliar com os prontuários, **Darlei** pela seleção dos blocos.

A **Imuno-histoquímica** por ter me apresentado com grandes amigas: **Elaine Keid**, **Elaine Gouveia**, **Flávia Gomes** e **Ana Paula**.

Aos meninos do **Laboratório de Patologia do Hospital Sírio Libanês**, **Felipe**, **Laisaac**, **Fernando**, **Robson**, **Alexandre**, **Agenor**; e as meninas também, **Rosangela**, **Mayra**, **Márcia**, **Rosineide**, **Dra. Fernanda**, por dividirem comigo seu espaço e sua simpatia, em especial ao **Isaque Santana** e a **Josiane Costa** pela atenção, companhia e disposição.

As **Profas. Dras. Patrícia Maluf Cury** e **Kátia Ramos Moreira Leite** pelo diagnóstico patológico das amostras e auxílio nas análises de imuno-histoquímica.

Ao **Prof. Dr. Sebastião Roberto Taboga e Luis Roberto Faleiros** pela colaboração no preparo das lâminas e fotodocumentação.

Ao **Setor de Endoscopia do Hospital de Base, Dr. Kenji Miyazaki e Dr. Henrique Oliveira** pela coleta das biópsias de megaesôfago.

A **Profa. Dra. Agnes C. Fett-Conte, Adriana Barbosa e Cristina** do **Laboratório de Genética, FAMERP**, pela disponibilização do microscópio de fluorescência;

A **Profa. Dra. Eny Maria Goloni Bertollo, a secretária Eliana, Silvia e João (SAME)** pelo auxílio na consulta de prontuários;

Margaret Skokan, Pornthip Kiatsimkul, Sujatha Gajapathy e Yun Xiao, “Leila’s Angels”, pela convivência, ensino-aprendizagem, intercâmbio cultural, horários de almoço, horários extraordinários, caronas, confidências, confiança, segredos, desabafos, choros, ombros, caixinhas de lenços, abraços, alegrias, chocolates, sorvetes e principalmente pela amizade.

A equipe do **Cancer Center, University of Colorado Health Sciences Center**, pela convivência harmoniosa, agradável e muito produtiva, em especial a **Maria Pia Morelli, Marialuisa**

Valente, Sharvari Gayal, Christopher Koch e Efang Li pela amizade construída.

Ao **Prof. Dr. Antonio José Manzato** pela assessoria estatística.

A **Seção de Pós-graduação**, em especial a **Rosana Ferro, Rosemar Brena e Silvia Kazama**, por estarem sempre solícitas.

“Cada um que passa em nossa vida, passa sozinho, pois cada pessoa é única, e nenhuma substitui a outra. Cada um que passa em nossa vida, passa sozinho, mas não vai só, nem nos deixa só, leva um pouco de nós mesmos, e deixa um pouco de si mesmo. Há os que levam muito, mas há os que não deixam nada. Essa é a maior responsabilidade de nossa vida. É a prova evidente que duas almas não se encontram ao acaso” (*Saint – Exupéry*)

Ana Lúcia Dias, Ana Paula Moreno, Bárbara Bisoli, Brett Radetsky, Christina Mailloux, Cínara Brandão, Claudio Cordioli, Débora Santana, Eliza Oliveira, Fabián Mendiburu, Fernando Lago, Flávia Paba, Helena Lelis, Jozélia Ferreira, Junior Caixeta, Lauriane Giselle, Lidiane Moura, Profa. Dra. Maria Tercília A. Oliveira, Mário Sérgio Mantovani, Monique Beaudoin, Nancy Joleon, Hangeseth's, Montoya's, Paulsen's, Família Tinoco, Família Monteiro Amaral, Família Guerreiro, Família Farias Nóbrega; Sandra, Edimilson e Maryanne Melo; Nathalie, Nayara, Silvana e José Carlos de Souza; Nélio e Márcio Farias; Enéas e Mathias Bontempo; Jociléa, Alex e A.J.

Scalisse; Fatinha e Grazielle Prado; Cybelle, Maria Helena e Hindemburgo Almeida.

“Mas a Escola é a luz que ilumina o caminho da gente” (*Carlos Pedro Ferreira*)

A Universidade Estadual Paulista Júlio de Mesquita Filho – **UNESP**, ao Instituto de Biociências Letras e Ciências Exatas – **IBILCE**, ao Programa de Pós-Graduação em Genética – **PPGGen**, pela oportunidade de concluir esse passo tão importante em minha formação acadêmica.

“Dos filhos deste solo és mãe gentil
Pátria amada
Brasil” (*Joaquim Osório Duque Estrada*)

Aos órgãos de fomento: **CNPq** pela bolsa de estudos, **CAPES** pela bolsa de estágio de doutoramento no exterior e **FAPESP** pelo auxílio financeiro.

A todos aqueles que de maneira direta e indireta colaboraram para o desenvolvimento deste trabalho.

MUITO OBRIGADA

Epígrafe

“Você não sabe o quanto eu caminhei

Prá chegar até aqui

Percorri milhas e milhas antes de dormir

Eu não cochilei

Os mais belos montes escalei

Nas escuras de frio chorei

A vida ensina e o tempo traz o tom

Pra nascer uma canção

Com a fé do dia-a-dia

Encontrar solução

...

Meu caminho só meu Pai pode mudar”

(Toni Garrido, Lazão, Da Gama e Bino Farias)

Sumário

SUMÁRIO

I. INTRODUÇÃO.....	38
I.1. Lesões Benignas do esôfago.....	39
I.2. Carcinogênese do esôfago e alterações genéticas.....	45
I.3. Alterações Genéticas em esofagite crônica e megaesôfago	53
I.4. Controle de Proliferação Celular e Apoptose	56
II. OBJETIVOS	62
CAPÍTULO I: Genomic imbalances in esophageal squamous cell carcinoma identified by molecular cytogenetic techniques	64
CAPÍTULO II: p53, p16 and Fhit Proteins Expressions in Chronic Esophagitis and Chagas Disease	94
CAPÍTULO III: Expression of caspase-3 protein and Ki67 antigen in benign lesions and esophageal carcinoma and relationship with p53 protein.	104
CAPÍTULO IV: Chromosomal imbalances are uncommon in Chagasic megaesophagus	125
III. DISCUSSÃO.....	159
IV. CONCLUSÕES	167
REFERÊNCIAS BIBLIOGRÁFICAS.....	170
APÊNDICE	192
Apêndice 1: Termo de Consentimento Livre e Esclarecido (Sanduíche)	193
Apêndice 2: Questionário.....	194
Apêndice 3: Caracterização das amostras de mucosa esofágica normal e Carcinoma de células escamosa de esôfago.....	195
Apêndice 4: Caracterização das amostras de pacientes com esofagite crônica	196
Apêndice 5: Caracterização das amostras de pacientes chagásicos sem megaesôfago.....	197
Apêndice 6: Caracterização das amostras de pacientes chagásico com megaesôfago Apêndice.....	198
Apêndice 7: Chromosomal Mapping for the newly developed probes.	201
ANEXOS	202
Anexo 1. Parecer Consubstanciado do Comitê de Ética em Pesquisa Institucional (Projeto de Doutorado Original).....	203
Anexo 2. Parecer Consubstanciado do Comitê de Ética em Pesquisa Institucional (Projeto de Estágio de Doutorado no Exterior).	204
Anexo 3. Metodologias para o desenvolvimento de sondas utilizadas na técnica de Hibridação In Situ Fluorescente (FISH).....	205

Lista de Figuras

Lista de Figuras

I. INTRODUÇÃO

Figura 1. Imagens endoscópicas: (A) Esôfago Normal, (B) Esofagite Crônica.....	40
Figura 2. Esquema de alterações em megaesôfago: dilatação do corpo esofágico e falta de abertura do esfíncter esofagiano inferior.....	42
Figura 3. Vias intrínseca e extrínseca de apoptose.....	59

Capítulo I

Figure 1 Summary of copy number alterations. in esophageal squamous cell carcinomas analyzed by comparative genomic hybridization. tumor.....	93
---	----

Capítulo II

Figure 1. Immunohistochemical stain in esophageal mucosa.....	101
---	-----

Capítulo III

Figure 1. Immunostaining of Ki67 antigen and CPP32 in esophageal mucosa.....	124
--	-----

Capítulo IV

Figure 1. Exploratory analysis using three-dimensional plots of the percentages of loss genes status for individual patients is illustrated.....	155
Figure 2. Exploratory analysis using three-dimensional plots of the percentages of gain and loss genes status for individual patients is illustrated.....	156
Figure 3: Exploratory analysis using three-dimensional plots of the percentages of gain genes status for individual patients is illustrated.....	157
Figure 4. FISH images for the unbalanced genes and centromeric targets in the megaesophagus specimens.....	158

Lista de Tabelas

Lista de Tabelas

Capítulo II

Table I. Demographic and clinicopathological distribution, immunohistochemical and statistical analysis for the proteins p53, p16 and Fhit in esophageal mucosa	99
Table II. Kappa test to describe the intensity of agreement among the expression of three proteins simultaneously and association between protein expression, two by two, by Fisher's exact test on esophageal mucosa, in each evaluated group.....	100

Capítulo III

Table 1. Immunohistochemical and statistical analysis for Ki67 antigen and CPP32 protein in the different groups.....	120
Table 2. Association between expression of Ki67 antigen and CPP32 protein, by Fisher's Exact Test ($p < 0.05$).....	121
Table 3. Association between expression of p53 protein ^[15] with Ki67 antigen and CPP32 protein, by Fisher's Exact Test ($p < 0.05$).....	122

Capítulo IV

Table 1. Mean copy number per cell and standard deviation (SD) for the 12 DNA targets tested in esophageal mucosa, including 9 genes and 3 centromeric sequences.....	149
Table 2. Comparison among the patient groups based on total counting results for the targets.....	150
Table 3. Comparison between gene and centromere target.....	150
Table 4. Mean copy number per cell and ranging of each target in distinct grades of megaesophagus.....	151
Table 5. Frequencies of loss, balanced and gain of the targets in chagasic megaesophagus (CM) and health patients (NM).....	152

Table 6. Frequencies of nuclei with aneusomies of 3, 7 and 9 centromeres in
the different groups 153

*Lista de abreviaturas e
símbolos*

Lista de abreviaturas e símbolos

-	Absence of immunostain
+	Weak immunostain
++	Moderate immunostain
+++	Strong immunostain
$\bar{x}$	Mean
*	Statistically significant, $p < 0.05$
a, b, c	Statistically significant , $p < 0.05$
AAA	achalasia, adrenocortical insufficiency, alacrimia
aCGH	arranjos de CGH (array CGH)
<i>ADAM8</i>	ADAM metallopeptidase domain 8
AgNOR	Argyrophilic nucleolar organizer region
Ala	aminoácido Alanina
ANOVA	Analysis of Variance
<i>APAF-1</i>	apoptotic peptidase activating factor 1
Arg	aminoácido Arginina
B	Basal layer of tissue
BAC	Bacterial artificial chromosome
Bad	proteína Bad
Bak	proteína Bak
<i>BCL2</i>	B-cell CLL/lymphoma 2
Bcl-2	proteína Bcl-2 (Bcl-2 protein)
Bcl-X	proteína Bcl-X (Bcl-X protein)

<i>BRCA2</i>	breast cancer 2, early onset
Cat. #	catalog number
<i>CCND1</i>	cyclin D1
CD	Chagas Disease
<i>CDC25B</i>	cell division cycle 25 homolog B (S. pombe)
<i>CDK4</i>	cyclin-dependent kinase 4
<i>CDK6</i>	cyclin-dependent kinase 6
<i>CDKN2A</i> (<i>P16, INK4A, MTS-1</i>)	cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)
CE	Chronic Esophagitis
CGH	Comparative Genomic Hybridization
<i>cIAP1, 2</i>	baculoviral IAP repeat-containing 2
CK4	Cytokeratin 4
CK14	Cytokeratin 14
CONEP	Comitê Nacional de Ética em Pesquisa
CM	Chagasic Megaesophagus
CPP32	caspase 3, apoptosis-related cysteine peptidase
<i>DAB</i>	3,3'-diaminobenzidine
DAPI	4', 6'-diamino-2-phenylindole
DR	Receptores de Morte (Death Receptors)
<i>ECT2</i>	epithelial cell transforming sequence 2 oncogene
EGF	epidermal growth factor (beta-urogastrone)

<i>EGFR</i>	epidermal growth factor receptor [erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian])
<i>ERBB2</i>	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene
ESCC	Esophageal Squamous Cell Carcinoma
<i>FAD</i>	presenilin 1
<i>FADD</i>	Fas (TNFRSF6)-associated via death domain
FFPE	formalin fixed, paraffin embedded
<i>FGF3</i> (<i>INT-2</i>)	fibroblast growth factor 3 (murine mammary tumor virus integration site (v-int-2)
<i>FGF4</i>	fibroblast growth factor 4
<i>FGFR1</i>	fibroblast growth factor receptor 1
<i>FHIT</i>	fragile histidine triad gene
Fhit	proteína Fhit
FISH	Fluorescence <i>In Situ</i> Hybridization
FITC	Fluorescein microscopy filter
FRA	Região de sítio frágil
FRA3B	fragile site, aphidicolin type, common, fra(3)(p14.2)
H ₂ O ₂	Peróxido de Hidrogênio
HE	Coloração Hematoxilina & Eosina
<i>HGF</i>	hepatocyte growth factor (hepapoietin A;

	scatter factor)
<i>HGF-R</i>	hepatocyte growth factor receptor
HLA	Human Leukocyte Antigen
<i>IAP</i>	baculoviral IAP repeat-containing
<i>ILP2</i>	baculoviral IAP repeat-containing 8
INCA	Instituto Nacional do Câncer
Ki67	Antígeno Ki67
KYSE	Serie of esophageal squamous cell carcinoma cell line
LI	Labeling Index
LOH	Loss of Heterozygosity
<i>LY6K</i>	lymphocyte antigen 6 complex, locus K
mCGH	metaphase CGH
M-FISH	Multiplex fluorescence in situ hybridization
<i>MIB</i>	mindbomb homolog
MLPA	Multiplex Ligation Dependent Probe Amplification
<i>MYC</i>	v-myc myelocytomatosis viral oncogene homolog (avian)
<i>MYEOV</i>	myeloma overexpressed (in a subset of t(11;14) positive multiple myelomas)
n	number of specimens positive immunostained
N	Total of specimens
<i>NAIP</i>	NLR family, apoptosis inhibitory protein

NCL-Ki67p	monoclonal antibody Ki-67
<i>NCOA3</i>	nuclear receptor coactivator 3
NM	Normal mucosa, health patients
NP	it was not performed
ns	not significant, $p > 0.05$
oligo-array	Oligonucleotide array
p15	proteína p15
<i>P15</i> (<i>CDKN2B</i> , <i>INK4B</i> , <i>MTS-2</i>)	cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)
p16	proteína p16
p53	proteína p53
PCNA	proliferating cell nuclear antigen
PCR-SSCP	polymerase chain reaction-single strand conformation polymorphism
<i>PIK3CA</i>	phosphoinositide-3-kinase, catalytic, alpha polypeptide
<i>PPFIA</i>	protein tyrosine phosphatase, receptor type, f polypeptide (PTPRF), interacting protein (liprin)
pRb	proteína retinoblastoma
Pro	aminoácido Prolina
<i>RB</i>	retinoblastoma
<i>RB1</i>	retinoblastoma 1
RT	Room temperature

S	Superficial layer of tissue
SD	Standard Deviation
SE	Standard Error
<i>SHANK2</i>	SH3 and multiple ankyrin repeat domains 2
<i>SKY</i>	Spectral Karyotyping
<i>Smac/Diablo</i>	diablo homolog (Drosophila)
SNP	Single Nucleotide Polymorphism
SSC	Sodium chloride, sodium citrate solution
<i>SST</i>	somatostatin
TE	Serie of esophageal squamous cell carcinoma cell line
<i>TERC</i>	telomerase RNA component
<i>TFRC</i>	transferrin receptor (p90, CD71)
TGF α	transforming growth factor, alpha
<i>TMEM16A</i>	anoctamin 1, calcium activated chloride channel
<i>TP53</i>	tumor protein p53
<i>TP63</i>	tumor protein p63
vs	<i>versus</i>
v/v	Volume/volume
<i>XIAP</i>	X-linked inhibitor of apoptosis
<i>WT1</i>	Wilms tumor 1
<i>YAMA</i>	Aliase of caspase 3, apoptosis-related cysteine peptidase

YES-1

v-yes-1 Yamaguchi sarcoma viral oncogene
homolog 1

μ

mean

Resumo

RESUMO

O carcinoma de esôfago apresenta um modelo de progressão tumoral a partir da seqüência esofagite, atrofia, displasia, carcinoma *in situ* e carcinoma invasivo, com algumas alterações genéticas bem estabelecidas nos estágios iniciais e avançados da carcinogênese. Contudo em lesões benignas precursoras como o megaesôfago e esofagite crônica os estudos genéticos são escassos. Portanto, com o objetivo de identificar o envolvimento de algumas proteínas que participam da regulação do ciclo celular e apoptose, no presente estudo foi avaliada a expressão das proteínas p53, p16, Fhit, caspase-3 e do antígeno Ki67, por imuno-histoquímica. Foram utilizados cortes histológicos de mucosa de pacientes chagásicos crônicos sem (CD) e com megaesôfago (CM), este último grupo por apresentar maior risco de desenvolvimento de carcinoma esofágico, e pacientes com esofagite crônica (CE), devido à relação entre o processo inflamatório e carcinogênese. Estas amostras foram comparadas com carcinoma de células escamosas de esôfago (ESCC) e mucosa esofágica histologicamente normal (NM). Também se avaliou a ocorrência de concordâncias utilizando o Teste Kappa, entre os casos com a expressão alterada das proteínas nos diferentes grupos, assim como a ocorrência de associações, entre padrões alterados de expressão das proteínas com sexo, idade, hábitos tabagistas e etilistas. Outro objetivo do estudo foi avaliar o padrão de perdas e ganhos cromossômicos de genes freqüentemente descritos como relacionados com a carcinogênese esofágica, *FHIT*, *TP63*, *PIK3CA*, *EGFR*, *FGFR1*, *MYC*, *CDKN2A*, *YES1*, *NCOA3*, e centrômeros 3, 7 e 9, como controles, por FISH. A avaliação imuno-histoquímica revelou que a proporção de casos positivos para a proteína p53 aumentou progressivamente de acordo com a severidade da lesão, CD (7,7%), CM (26,1%), CE (52,2%) and ESCC (100%). Entretanto, as proteínas p16 e Fhit não mostraram diferenças estatisticamente significantes entre os grupos, mas também, em CE observou-se um número maior de casos com expressão reduzida dessas proteínas (95,7%; 34,8%, respectivamente), similar ao ESCC (90,9%; 18,2%, respectivamente). A análise de concordância entre as expressões das proteínas (Teste Kappa) não evidenciou concordâncias entre os casos com expressão alterada e normal, para as três proteínas simultaneamente. Porém, o Teste Exato de Fisher mostrou associação entre a expressão alterada das proteínas p53 e p16; exceto em ESCC, único grupo que apresentou associação entre p53 e Fhit. Enquanto a associação entre a expressão alterada de p16 e Fhit, só não foi evidenciada em NM. A investigação de alterações nas taxas de proliferação celular e apoptose evidenciaram que o índice de marcação de Ki67 (LI) foi similar em todos os grupos, 30,1% em NM, 30,9% em CD, 42,3% em CM; 44,6% em CE e 48,4% em ESCC, no total de 500 células analisadas por caso, não sendo observadas diferenças significantes. A frequência de positividade de CPP32 foi similar nos grupos CD (30,8%), CM

(30,4%) e CE (34,8%), mas aumentada em ESCC (55,5%). Também não foi observada associação entre os níveis de CPP32, Ki67 e expressão da proteína p53 nos diferentes grupos, nem dessas com os parâmetros de idade, sexo e hábitos tabagista e etilista. A análise citogenética para avaliação dos padrões de perdas e ganhos nas camadas superficial e basal de mucosa esofágica não mostrou diferenças significantes para a maioria dos alvos investigados, exceto para *EGFR* no grupo CM, que apresentou frequência menor de cópias na camada basal. Considerando-se o número total de cópias (camadas superficial e basal) apenas o gene *CDKN2A* e centrômero 9 apresentaram médias significativamente menores em CM que em NM. Enquanto, quando foram avaliadas, para cada gene, as frequências de núcleos com padrão de perda ou ganho, apenas o gene *PIK3CA* apresentou frequência significativamente maior de núcleos com perdas no grupo CM em comparação a NM. A partir de análise exploratória usando “plots” tridimensional das porcentagens das classes de genes com perda ou ganho, alguns casos de CM mostraram níveis, não-estatisticamente significantes, mas que se destacaram da frequência média do grupo, de perdas para genes específicos: *TP63*, *FHIT*, *PIK3CA*, *EGFR*, *CDKN2A*, *YES1*; CEP9 ou de ganhos para *PIK3CA*, *TP63*, *FGFR1*, *MYC*, *CDKN2A*, *NCOA3*, e *CEP9*, sendo a maioria associada aos graus III e IV do megaesôfago, provavelmente devido ao processo inflamatório acentuado. Em conclusão, o presente estudo evidência o envolvimento da expressão aumentada da proteína p53 nos processos iniciais da carcinogênese esofágica (esofagite crônica e megaesôfago chagásico), sugerindo sua utilização como marcador em lesões precursoras. De modo contrário, perdas na expressão de Fhit e p16 não parecem ser significantes, assim como alterações na expressão do antígeno Ki67 e da proteína caspase-3, sugerindo que não há evidências de alterações importantes nos níveis de proliferação celular e apoptose nas lesões benignas e carcinoma, nas condições estudadas. Além desses dados, observação de que regiões genômicas com desequilíbrios na carcinogênese esofágica não são significativamente afetadas em megaesôfago chagásico sugere que não será possível utilizar tais características como marcadores eficazes de risco para desenvolvimento de ESCC em megaesôfago.

Palavras-chave: Megaesôfago chagásico, Esofagite crônica, Carcinoma de células escamosas, apoptose, proliferação celular, ganhos, perdas e amplificações gênicas, proteínas reguladoras do ciclo celular

Abstract

ABSTRACT

Esophagus carcinoma presents a tumor progression model from the sequence esophagitis, atrophy, dysplasia, carcinoma *in situ* and invasive carcinoma, with some well-established genetic changes in early and advanced stages of carcinogenesis. In benign precursor lesions such as megaesophagus and chronic esophagitis genetic studies are scarce. Therefore, to identify the involvement of certain cell cycle and apoptosis regulatory proteins, the present study evaluated the expression of p53, p16, Fhit, caspase-3 and Ki67 antigen by immunohistochemistry. Histological sections of esophageal mucosa were obtained from chronic chagasic patients without (CD) and megaesophagus (CM), the latter group presents higher risk of developing esophageal cancer, and patients with chronic esophagitis (CE), because the relationship between the inflammatory process and carcinogenesis. These samples were compared with squamous cell carcinoma of the esophagus (ESCC) and histologically normal esophageal mucosa (NM). It also assessed the occurrence of agreement using the Kappa test, among the cases with altered expression of proteins in different groups as well as the occurrence of associations, and altered patterns of protein expression with sex, age, smoking and alcohol habits. Another aim of the study was to evaluate the pattern of chromosomal gains and losses of genes frequently described as related to esophageal carcinogenesis, *FHIT*, *TP63*, *PIK3CA*, *EGFR*, *FGFR1*, *MYC*, *CDKN2A*, *YES1*, *NCOA3* and centromere 3, 7 and 9, as controls for FISH. The immunohistochemical evaluation showed that the proportion of cases positive for p53 protein increased progressively according to the severity of the injury, CD (7.7%), CM (26.1%), CE (52.2%) and ESCC (100%). However, the proteins p16 and Fhit showed no statistically significant differences between groups, but also in CE was observed a greater number of cases with reduced expression of these proteins (95.7%, 34.8%, respectively), similar to ESCC (90.9%, 18.2%, respectively). The analysis of correlation between the expressions of proteins (Kappa test) showed no correlation between the cases with normal expression changed and, for the three proteins simultaneously. However, the Fisher's exact test showed an association between the altered expression of p53 and p16, except in ESCC, the only group that showed an association between p53 and Fhit. While the association between altered expression of p16 and Fhit, was not observed only in NM. Investigation of changes in cell proliferation and apoptosis rates revealed that the rate of labeling of Ki67 (LI) was similar in all groups, 30.1% in NM, 30.9% in CD, 42.3% in CM; 44.6% in CE and 48.4% in ESCC, in total 500 cells examined per case, no significant differences were observed. The frequency of positivity of CPP32 was similar in the groups CD (30.8%), CM (30.4%) and CE (34.8%) but increased in ESCC (55.5%). Also no association was found between the levels of CPP32, Ki67 and p53 protein expression in different groups, or those with the parameters such as age,

sex, smoking and alcohol habits. The cytogenetic analysis to assess patterns of losses and gains in the superficial and basal layers of esophageal mucosa showed no significant differences for most of the targets investigated, except for *EGFR* in the CM group, which showed lower frequency of copies in the basal layer. Considering the total number of copies (superficial and basal layers) only the *CDKN2A* gene and centromere 9 showed significantly lower averages in CM than in NM. Meanwhile, they were evaluated for each gene, the frequencies of nuclei with standard loss or gain, only the *PIK3CA* gene showed a significantly higher frequency of nuclei with losses in the CM group compared to NM. From exploratory analysis using three-dimensional plots of the percentages of genes classes with loss or gain, some cases of CM showed levels, non-statistically significant, but it highlighted the group's average frequency of losses for specific genes: *TP63*, *FHIT*, *PIK3CA*, *EGFR*, *CDKN2A*, *YES1*; CEP9 or gains for *PIK3CA*, *TP63*, *FGFR1*, *MYC*, *CDKN2A*, *NCOA3* and CEP9, most of them associated with grades III and IV of megaesophagus, probably due to the inflammatory process. In conclusion, this study highlighted the involvement of increased expression of p53 protein in cases of benign precancerous lesions (esophagitis and chronic chagasic megaesophagus), suggesting its use as a marker in precursors lesions. Controversially, losses on the expression of *Fhit* and p16 not appear to be significant as well as changes in the expression of Ki67 antigen and caspase-3 protein, suggesting that there is no evidence of major changes in the levels of cell proliferation and apoptosis in carcinoma and benign lesions under the studied conditions. Besides these data, the findings that genomic imbalances common in esophageal carcinomas are not affected in megaesophagus suggest that these features will not be effective biomarkers for risk assessment of esophageal carcinoma in chagasic megaesophagus.

Key words: chagasic megaesophagus, chronic esophagitis, esophageal squamous cell carcinoma, apoptosis, cell proliferation, genomics imbalances, cell cycle regulators proteins

Introdução

I. INTRODUÇÃO

1.1. Lesões Benignas do esôfago

O esôfago, devido ao seu papel na condução de substâncias até o estômago, está exposto a condições adversas responsáveis pela ocorrência de lesões que variam desde uma simples azia, esofagites e hérnias de hiato até cânceres letais. As lesões benignas mais frequentemente encontradas neste órgão são as esofagites e a acalasia ou megaesôfago (BRASILEIRO FILHO, 2006).

A esofagite é uma lesão da mucosa esofágica com subsequente inflamação, mundialmente comum. Nos Estados Unidos e em outros países ocidentais, a esofagite ocorre em aproximadamente 5% da população adulta e prevalências maiores são encontradas no Irã e algumas partes da China (CRAWFORD, 2004).

As esofagites podem ocorrer na forma aguda e crônica, sendo a primeira causada pelo refluxo do conteúdo gástrico, infecção por bactérias, vírus ou fungos, ingestão de alimentos e líquidos excessivamente quentes, substâncias cáusticas (BRASILEIRO FILHO, 2006) e ingestão de medicamentos (MINCIS, 1997).

A esofagite crônica (Figura 1) pode ser provocada pelos mesmos agentes que a aguda e ainda pela estase alimentar (retenção de alimentos) (CRAWFORD, 2004; BRASILEIRO FILHO, 2006). Essa estase pode causar a esofagite crônica devido ao contato prolongado do alimento parcialmente digerido com a mucosa esofágica, aumento do crescimento bacteriano e irritação química (RIBEIRO *et al.*, 1996). Quanto maior o grau da dilatação esofágica, maior a proliferação de microorganismos no

líquido de estase, contribuindo para o desenvolvimento da inflamação (PAJECKI *et al.*, 2002). As alterações fundamentais das esofagites são: infiltrado inflamatório de mononucleares, hiperplasia do epitélio, erosões e ulcerações da mucosa, estenose do órgão, hiperemia, friabilidade e graus variados de fibrose na parede (CRAWFORD, 2004; MADER *et al.*, 2002; BRASILEIRO FILHO, 2006).

Essas duas principais lesões do esôfago, ou seja, o refluxo esofágico e a esofagite crônica estão associados a carcinogênese esofágica. A primeira, possivelmente, aumenta a susceptibilidade ao desenvolvimento de esôfago de Barret e conseqüentemente ao adenocarcinoma (JONES *et al.*, 2003), enquanto que a esofagite crônica é considerada uma alteração precoce no carcinoma de células escamosas do esôfago (MANDARD *et al.*, 1998).

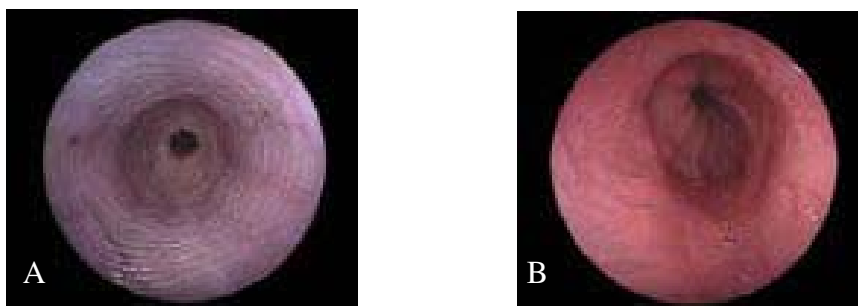


FIGURA 1. Imagens endoscópicas: (A) Esôfago Normal, (B) Esofagite Crônica (IQB, Instituto Químico Biológico, 2007)

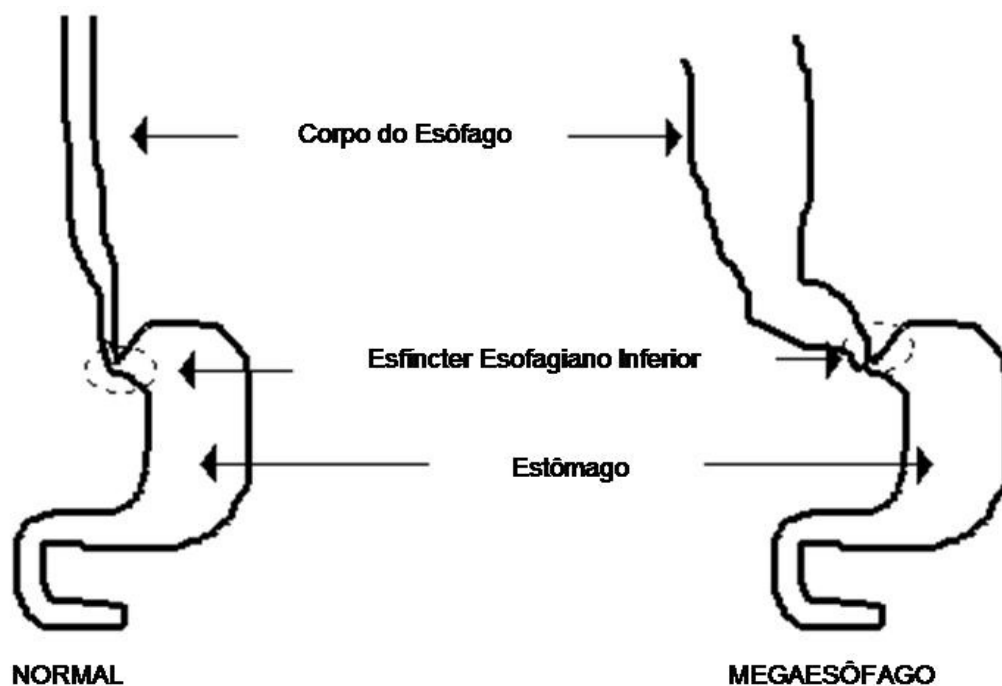
Exames endoscópicos em grupos de alto risco de desenvolvimento de câncer, de diferentes países, têm demonstrado a presença de esofagite crônica em 42% a 84% dos indivíduos, atrofia em 3,8% a 10% e displasia em 2,4% a 8% (CASTELLETTO *et al.*, 1992). Estes dados sugerem que a história natural do carcinoma esofágico pode iniciar-se a partir da esofagite (MUÑOZ, 1997). Pacientes com evidência histológica de esofagite desenvolveram, por um período de até 6,8 anos, carcinoma de células escamosas do esôfago e câncer esofágico não específico em 0,15% e 0,3% dos casos respectivamente, indicando um

risco moderadamente aumentado para o desenvolvimento de carcinoma, mas não para o adenocarcinoma (MURPHY *et al.*, 2005). Por outro lado, Wang *et al.* (2005) em acompanhamento clínico por endoscopia durante o período de 13 anos, não observaram evidências de que a presença de esofagite aumente o risco de desenvolvimento do carcinoma de esôfago.

Outra lesão do esôfago com potencial pré-canceroso é o megaesôfago ou dilatação do esôfago (Figura 2), também denominada acalasia, cardioespasmo e aperistalsia, (VANTRAPPEN; HELLEMANS, 1982 *apud* KRAICHELY; FARRUGIA, 2006). Ele é caracterizado pela destruição ou ausência dos plexos nervosos intramurais, determinando a ausência de peristaltismo ao nível do corpo do órgão e a falta de abertura do esfíncter esofágico inferior, em resposta à deglutição. Em consequência, inicia-se a retenção do bolo alimentar (estase esofágica) na luz do órgão (BRASILEIRO FILHO, 2006), pela dificuldade no relaxamento do esfíncter esofágico inferior (KRAICHELY; FARRUGIA, 2006). O sintoma clínico clássico do megaesôfago é a dificuldade para engolir (disfagia) que ocorre de modo progressivo, evoluindo de alimentos sólidos para pastosos, até a pessoa engolir apenas líquidos. Podem ocorrer também a regurgitação e a aspiração noturnas do alimento não digerido (OLIVEIRA *et al.*, 1998; CRAWFORD, 2004).

Mundialmente, tais características correspondem à lesão denominada de acalasia, cuja patogênese é pobremente conhecida e parece envolver alterações degenerativas na inervação neural levando à dilatação do órgão (CRAWFORD, 2004). A estase alimentar constitui um estímulo permanente para as contrações do corpo do esôfago na tentativa de ultrapassar o obstáculo representado pelo esfíncter. No início, essas contrações são fortes e irregulares levando à hipertrofia das fibras musculares e espessamento da camada muscular caracterizando, portanto, a fase inicial da dilatação (megaesôfago). As contrações tornam-se progressivamente menos intensas e as fibras musculares vão alongando-se e gradualmente sendo substituídas por tecido conjuntivo,

tornando a dilatação mais acentuada pela atonia completa da musculatura. Conseqüentemente, provoca a inflamação da mucosa (esofagite), que pode determinar o aparecimento de acantose, paraceratose e leucoplasia, lesões possivelmente pré-cancerosas (PINOTTI, 1996; KRAICHELY; FARRUGIA, 2006).



ERROR: stackunderflow
OFFENDING COMMAND: ~

STACK:

Objetivos

II. OBJETIVOS

Considerando que algumas alterações genéticas estão relacionadas às etapas iniciais da carcinogênese do esôfago, justifica-se a necessidade de estudos que avaliem alterações na expressão de proteínas e desequilíbrios gênicos em lesões benignas do esôfago com potencial pré-canceroso, sendo assim, o presente trabalho teve como objetivos:

- a. Avaliar a expressão das proteínas p53, p16 e Fhit, em mucosa esofágica de pacientes com lesões benignas como esofagite crônica e megaesôfago chagásico em comparação com o carcinoma de esôfago; assim como verificar a ocorrência de concordância e associações no padrão de expressão das três proteínas em lesões benignas e carcinoma esofágico;
- b. Investigar a ocorrência de alterações em apoptose, por meio da expressão da caspase-3, e proliferação celular, com o antígeno Ki67, e associações com a expressão da proteína p53 nos grupos de lesões benignas e carcinoma do esôfago;
- c. Avaliar no grupo de pacientes com megaesôfago chagásico os padrões de deleção e ganho de genes citados em desequilíbrios gênicos em carcinoma esofágico identificados a partir de dados da literatura, assim como associações com a graduação do megaesôfago;
- d. Investigar nos grupos de lesões benignas e carcinoma a ocorrência de associações entre a expressão das proteínas p53, p16, Fhit, caspase-3 e antígeno Ki67, assim como os desequilíbrios gênicos com parâmetros como idade, sexo, hábitos tabagista e etilista.

Capítulo I

CAPÍTULO I: Genomic imbalances in esophageal squamous cell carcinoma identified by molecular cytogenetic techniques

Authors: Marilanda Ferreira Bellini¹, Ana Elizabete Silva¹, Marileila Varella-Garcia²

Affiliations

¹ Laboratório de Citogenética e Biologia Molecular, Department of Biology, UNESP, São Paulo State University, Campus São José do Rio Preto, SP; Brazil

² Medicine/Medical Oncology, University of Colorado, Health Sciences Center, Aurora, Colorado, USA

Short running title: Genomic imbalances in esophageal cancer

Key words: Genomic Gains, Genomic Losses, Amplifications, Oncogenes, CGH, FISH and Esophageal Carcinoma

Corresponding author:

Ana Elizabete Silva,

IBILCE/UNESP – Departamento de Biologia, Rua Cristóvão Colombo, 2265, São José do Rio Preto, SP, Brazil - CEP. 15054-000; phone 55 - 17 – 32212384.

e-mail:anabete@ibilce.unesp.br

Artigo de revisão a ser submetido e redigido nas normas da revista

GENETICS AND MOLECULAR BIOLOGY (e-ISSN: 1678-4685)

Resumo

A presente revisão sumariza as alterações cromossômicas detectadas por técnicas de citogenética molecular em carcinoma de células escamosas de esôfago (ESCC), a nona neoplasia maligna mais comum no mundo. A análise genômica total em linhagens celulares de ESCC e em tumores primários indicou que as regiões de ganho mais freqüentes são observadas em regiões dos cromossomos 1, 2q, 3q, 5p, 6p, 7, 8q, 9q, 11q, 12p, 14q, 15q, 16, 17, 18p, 19q, 20q, 22q, e X, com amplificações focais em 1q32, 2p16-22, 3q25-28, 5p13-15.3, 7p12-22, 7q21-22, 8q23-24.2, 9q34, 10q21, 11p11.2, 11q13, 13q32, 14q13-14, 14q21, 14q31-32, 15q22-26, 17p11.2, 18p11.2-11.3, e em 20p11.2. As regiões de perdas recorrentes são 3p, 4, 5q, 6q, 7q, 8p, 9, 10p, 12p, 13, 14p, 15p, 18, 19p, 20, 22, Xp e Y. Ganhos em 5p e 7q, e deleções em 4p, 9p e 11q são fatores prognósticos significantes para estadiamento de ESCC. Ganhos em 6p e 20p, e perdas em 10p e 10q são as alterações mais significantes tanto em carcinomas primários quanto em metástases, o que sugere que essas regiões contenham oncogenes e genes supressores tumorais. Ganho em 12p e perda em 3p podem estar associados com rápida progressão da doença. Essas alterações genômicas podem representar marcadores de diagnóstico ou prognóstico aplicáveis na prática clínica, assim como identificarem moléculas alvo para terapia personalizada.

Palavras-chave: carcinoma de células escamosas de esôfago, deleções, ganhos, amplificações, citogenética molecular

Abstract

This review summarizes chromosome changes detected by molecular cytogenetic approaches in esophageal squamous cell carcinoma (ESCC), the ninth most common malignancy in the world. Whole genome analyses in ESCC cell lines and tumors indicated that the most frequent genomic gains were observed at 1, 2q, 3q, 5p, 6p, 7, 8q, 9q, 11q, 12p, 14q, 15q, 16, 17, 18p, 19q, 20q, 22q, and X, with focal amplifications at 1q32, 2p16-22, 3q25-28, 5p13-15.3, 7p12-22, 7q21-22, 8q23-24.2, 9q34, 10q21, 11p11.2, 11q13, 13q32, 14q13-14, 14q21, 14q31-32, 15q22-26, 17p11.2, 18p11.2-11.3, 20p11.2. The recurrent losses involved 3p, 4, 5q, 6q, 7q, 8p, 9, 10p, 12p, 13, 14p, 15p, 18, 19p, 20, 22, Xp and Y. Gains at 5p and 7q, and deletion at 4p, 9p, and 11q were significant prognostic factors for patients with ESCC. Gains at 6p and 20p, and losses at 10p and 10q were the most significant imbalances, both in primary carcinoma and in metastasis, suggesting these regions could harbor oncogenes and tumor suppressor genes. Gains at 12p and losses at 3p could be associated with poor relapse-free survival. These changes may represent markers with clinical applicability at the diagnosis or prognosis or may identify molecular targets for personalized therapy.

Key words: esophageal squamous cell carcinoma, deletion, gain, amplification, molecular cytogenetic

Esophageal Carcinoma

Cancer of the esophagus has been reported as the ninth most common malignancy in the world, but its incidence varies largely among regions (Lam *et al.*, 2000), with higher incidence in China, Japan, Singapore and Puerto Rico (INCA, 2008). The American Cancer Society estimated around 16,470 new cases of esophageal carcinoma in the USA population for 2008 (American Cancer Society, 2008). The Brazilian National Institute of Research in Cancer (INCA) reported that esophageal cancer was the sixth in the cancer rank of mortality in 2000 with 5,307 deaths (INCA, 2008) and estimated about 10550 new cases in 2008 ranging among geographical areas from 1.04 to 19.07 in males and from 0.39 to 7.58 in 100,000 in females.

The development of human esophageal cancer is a progressive process, resulting in successive accumulation of genetic changes that culminate in the malignant transformation (Knudson, 1985; Somers; Schechter, 1992; Xue *et al.*, 2006). An early indicator of this process is the increased proliferation of esophageal epithelial cells, morphologically evolving to stages of basal cell hyperplasia, dysplasia, carcinoma *in situ* and invasive carcinoma (Muñoz, 1997; Mandard *et al.*, 2000; D'Amico, 2006).

With the exception of the USA the esophageal squamous cell carcinoma (ESCC) is the prevalent histological type worldwide (except in USA) and has multifactorial origin. Besides environmental components (Crawford, 2004), several genetic factors are associated with esophageal carcinogenesis such as chromosomal aneuploidy, allelic deletions, activation of oncogenes and inactivation of tumor suppressor genes (Kuwano *et al.*, 2005). At the cellular level, these factors head to disorders of the cell proliferation, differentiation and apoptosis (Koch *et al.*, 1994; McCabe; Dlamini, 2005; D'Amico, 2006; Daigo; Nakamura, 2008; Khushalani, 2008).

Specific chromosome aberrations have been identified as markers for diagnosis and prognosis in solid tumors (Tada *et al.*, 2000; Yen *et al.*, 2000; Shiomi *et al.*, 2003; Qin *et al.*, 2004, 2005ab; Wang *et al.*, 2006; Qin *et al.*, 2008). Despite reports of numerous chromosome changes, no particular diagnostic or prognostic chromosomal markers have been described in esophageal carcinoma. The aim of this review is to summarize the recurrent chromosomal changes detected by molecular cytogenetic approaches in ESCC, such as gains and losses in regions that may harbor oncogenes and tumor suppressor genes. These chromosomal imbalances may represent markers with clinical applicability in the diagnosis or prognosis or be supportive of novel targeted therapies.

Molecular Cytogenetic Technologies Identify Genomic Changes in Cancer

The primary cytogenetic technique used for a better understanding of the molecular pathogenesis involved in esophageal carcinogenesis was DNA fluorescence *in situ* hybridization (FISH). FISH uses small fragments of DNA as fluorescent probes that bind to chromosomal sequences of the target DNA to which they show a high degree of homology (Bauman *et al.*, 1980; Langer *et al.*, 1981) it detects and localizes specific DNA sequences on chromosomes. FISH probes are often derived from fragments of DNA on the order of few hundreds to 200 thousand base-pairs that are isolated, purified, amplified and labeled with fluorochrome-conjugated nucleotides. The DNA probe may be hybridized to distinct DNA targets such as metaphase chromosomes, interphase nuclei, and extended chromatin fibers and DNA fragments in a variety of biological specimens or platforms, including isolated cells, tissue section and oligonucleotide arrays (Speicher; Carter, 2005) and DNA microarrays (Solinas-Toldo *et al.*, 1997; Pinkel *et al.*, 1998).

There are numerous variants of the FISH assay; the variant most effectively used to detect genome wide imbalances is called Comparative Genomic Hybridization (CGH). CGH was originally developed as the competitive hybridization of a mix of the test DNA and a normal reference DNA, labeled with different fluorochromes, to metaphase spreads of a normal specimen (metaphase CGH - mCGH) (Kallioniemi *et al.*, 1992; du Manoir *et al.*, 1993).). Chromosomal regions of the test DNA with normal copy numbers will show a balanced ratio of hybridization with the test and the control DNA. Chromosomal regions with excess or lack of copy numbers in the test DNA will show the predominant color of the hybridization in the normal template. The DNA used in the assay is extracted from dividing or non-dividing cells from virtually all types of tissues even those fixed in formalin, thus the technique allows an informative overview of archived specimens. The level of resolution of specific imbalances results in the mCGH assay basically depends on the condensation of the chromosomes to which the DNA mix is hybridized and is estimated as ranging from 5 to 10 MB (Kallioniemi *et al.*, 1992; Speicher; Carter, 2005). It is possible to identify large regions involved in low level of genomic gain or loss and small regions with focal amplification but overall the resolution of the mCGH is limited or insufficient for identification of specific chromosomal bands (Kallioniemi *et al.*, 1992; Speicher; Carter, 2005). Therefore, mCGH studies are used as preliminary tools, to infer potential genes located in imbalanced regions and to support refined studies with higher resolution techniques, such as array CGH (aCGH) or FISH with single gene probes (Nakakuki *et al.*, 2002; Arai *et al.*, 2003)

The aCGH technique detects chromosome copy number changes at a much higher resolution level than mCGH (Solinas-Toldo *et al.*, 1997; Pinkel *et al.*, 1998). Instead of using metaphase spreads as template for hybridization, the aCGH uses a collection of DNA inserts contained in bacterial artificial chromosomes (BAC arrays) or oligonucleotides (oligo-arrays) printed on a glass slide. Similarly to the

mCGH, a differentially labeled mix of DNAs from the test sample and a normal reference control sample is hybridized with the platform and the ratio of the fluorescence intensity of the test to the reference DNA is calculated. Using aCGH, copy number changes can be detected at levels of few hundred kilobases of DNA sequences for BAC arrays or 30 kb for oligoarrays (Snijders *et al.*, 2001; Fiegler *et al.*, 2003; Medical Genetics Laboratories, 2008). SNP array is a type of DNA microarray which is used to detect polymorphisms within a population. A single nucleotide polymorphism (SNP), a variation at a single site in DNA, is the most frequent type of variation in the genome. For example, there are around 10 million SNPs that have been identified in the human genome. As SNPs are highly conserved throughout evolution and within a population, the map of SNPs serves as an excellent genotypic marker for research (Sherry *et al.*, 2001).

Other variants of the FISH technology are the Multiplex-FISH (M-FISH) and the Spectral Karyotyping (SKY). These procedures allow identification of the chromosomal origin of each chromosomal region in a metaphase cell, e.g., visualizes all 24 human chromosomes in a single hybridization (Speicher; Carter, 2005). In both techniques, the probe set is a pool of differentially labeled DNAs for each of the 24 human chromosomes. In the M-FISH, images for each fluorochrome are individually collected and merged, and a combinatorial labeling algorithm identifies each chromosome, visualized in pre-defined pseudocolour (Speicher *et al.*, 1996). In the SKY assay, a single image is captured per cell and an interferometer is used for discrimination of the fluorochrome spectrum in each image pixel, to which a pseudocolor is assigned (Schröck *et al.*, 1996). Both techniques have been successful in clarify complex chromosomal rearrangements in solid tumors (Schröck; Padilla-Nash, 2000), including ESCC (Yen *et al.*, 2003).

Altogether, the numerous variants of FISH technology have allowed accurate identification of DNA sequences of interest in the

chromosomes and search of the whole genome total for gains and losses associated with in the carcinogenesis process.

Genomic Imbalances in ESCC cell lines

ESCC lines are highly abnormal cytogenetically. An integrated study using mCGH, SKY and FISH with single probes was performed in 8 ESCC cell lines; the pooled CGH results revealed frequent gains on almost all chromosome arms (1p, 1q, 3q, 5p, 6p, 7p, 7q, 8q, 9q, 11q, 12p, 14q, 15q, 16p, 16q, 17q, 18p, 19q, 20q, 22q, and Xq), while frequent losses were found on 3p, 4, 5q, 6q, 7q, 9p, and 18q. SKY analyses detected 195 translocations, 13 deletions and 2 duplications. The most frequently amplified genes were *PIK3CA* and *TP63* (3p28). The protein encoded by *PIK3CA* represents the catalytic subunit, which uses ATP to phosphorylate phosphatidylinositol and *TP63* encodes, a protein that works in the development and maintenance of stratified epithelial tissues, these oncogenes, were amplified in 6 and 5 cell lines, respectively (Yen *et al.*, 2003; National Center for Biotechnology Information, 2009).

Multiple gains and losses involving different chromosomal regions were also found by mCGH in 10 ESCC cell lines of the KYSE series, TE-1–6, -8 – 11, -13, and -15. The most frequent losses were observed at chromosome arms 3p, 4p and q, 8p, 9p, 18q, and Xp; whereas the most common gains were noted at 1q, 3q, 5p, 7p, 8q, 9q, 11q, 18p, 20q and Xq. While focal loss was only identified at 11q 23-25, focal amplifications were detected at 1q32, 2p16-22, 3q25-28, 5p13-15.3, 7p12-22, 7q21-22, 8q23-24.2, 9q34, 10q21, 11p11.2, 11q13, 13q32, 14q13-14, 14q21, 14q31-32, 15q22-26, 17p11.2, 18p11.2-11.3, 20p11.2 (Shinomiya *et al.*, 1999; Pimkhaokham *et al.* 2000; Su *et al.*, 2006).

Later, Yang *et al.* (2008a) characterized cytogenetic abnormalities in the cell line KYSE 410-4 using M-FISH, detecting chromosome gains at 2q, 3, 8, 17p, and X. An isochromosome 3q was

visualized in this line, which might be one intermediate mechanism leading to 3p loss and 3q gain. For the cell line KYSE 180, M-FISH analysis detected loss of DNA copy number at 4p, 5q, 6q, 9, 10p, 12p, 13, 14p, 15p, 18p, 18q, 20, 22, and Y; chromosomal gains and translocations mainly involved chromosomes 1, 2p, 3, 4p, 5p, 5q, 6p, 7, 8, 10q, 11, 12q, 14q, 16, 17q, 19, and Xp. Seven derivative chromosomes involving chromosomes 5, 8, 12, 14, 14, 14, and 17 presented complex translocations, each involving three or four chromosomes and loss of chromosomes 9, 13, and Y were also detected (Wu *et al.*, 2006).

Since previous studies of CGH revealed frequent amplifications in 18p in esophageal cell lines (Shimada *et al.*, 1992; Pimkhaokham *et al.*, 2000), Nakakuki *et al.* (2002) screened 29 ESCC cell lines to discern amplifications by FISH of 14 known genes and 21 uncharacterized transcripts within amplicons in chromosome 18. These authors also investigated the levels of protein expression of these genes by southern-, dot- and northern-blotting. Only four known genes, showed amplification and correlated over-expression, *YES1*, *TYMS*, *HEC* and *TGIF*. *YES1* encodes a protein with tyrosine kinase activity, *TYMS* is critical for DNA replication and repair, *HEC* is involved in spindle checkpoint signaling and *TGIF* is a highly conserved transcription regulator with potential role in the transmission of nuclear signals during development and in the adult.. , These results suggest those genes are likely candidate targets for 18p11.3 amplification and may be associated with esophageal tumorigenesis.

In summary, these whole genome analyses in ESCC cell lines indicated that the most frequent genomic gains were observed at 1p, 1q, 2q, 3q, 5p, 6p, 7p, 7q, 8q, 9q, 11q, 12p, 14q, 15q, 16p, 16q, 17p, 17q, 18p, 19q, 20q, 22q, and X, with focal amplifications at 1q32, 2p16-22, 3q25-28, 5p13-15.3, 7p12-22, 7q21-22, 8q23-24.2, 9q34, 10q21, 11p11.2, 11q13, 13q32, 14q13-14, 14q21, 14q31-32, 15q22-26, 17p11.2, 18p11.2-11.3, 20p11.2. The recurrent losses involved 3p, 4, 5q, 6q, 7q, 8p, 9, 10p,

12p, 13, 14p, 15p, 18, 20, 22, Xp and Y (Figure 1A). The detected alterations affect most of the genome and involve regions harboring many known oncogenes and tumor suppressor genes, as well regions not yet associated with such genes. Although the level of molecular resolution of the majority of these studies is low and not conclusive, these findings are promising in assisting the design of refined investigations on the molecular pathogenesis and development of new therapeutics of ESCC.

Genomic Imbalances in ESCC tumor samples

Despite the better understanding of the risk factors and cellular derangements associated with esophageal cancer, the clinical treatment of this disease surprisingly has had little changes and long-term survival from esophageal cancer remains poor, with 5-years survival rates about 20% (Hsia *et al.*, 2003).

The combination of different techniques (FISH, mCGH and aCGH) indicated the chromosomes 1, 3, 7, 9, 11, 18, 19 and 20 shows high frequency of alterations. Genomic profiles in primary carcinomas have showed imbalances affecting most of the chromosomes, such as gains at 1q, 3q, 5p, 7p, 8q, 11q, 13q, 18p, 20q, Xq; and losses at 1p, 3p, 4p, 8p, 9p, 18q, 19, 22q and Y. Focal losses at 9p13, focal gains at the regions 5p15, 8p12-11.2, 8q24, 11q13, 14q32, and amplifications at 1p34, 2p24, 2q24-34, 3q22-ter, 7p12-22, 8q13-qter, 11p11.2, 11q13, 12p11.2, 13q21-34, 17q12, 20q12-13 and Xq27-28 are also commons findings (Shinomiya *et al.*, 1999; Pack *et al.*, 1999; Mayama *et al.*, 2000; Yen *et al.*, 2001; Kamitani *et al.*, 2002; Yen *et al.*, 2003; Kwong *et al.*, 2004; Qin *et al.*, 2004, 2005ab, 2008; Sugimoto *et al.*, 2007). High-level amplifications were observed in 30 regions and recurrently involved 7p11.2 and 11q13. *EGFR* is a cell surface protein that when binds to its ligand induces receptor dimerization and tyrosine autophosphorylation, leading cell proliferation the 2nd region harbors *CCND1* is a regulator of CDK kinases required for

G1/S cell cycle transition. (Carneiro *et al.*, 2008; National Center for Biotechnology Information, 2009). Interstitial deletions in 1p, 3p, 5q, 6q, 11q, and 12q were also detected (Pack *et al.*, 1999). These observations altogether evidenced that accumulation of chromosomal aberrations is common in ESCC (Figure 1B) and suggest that 1q, 3q, 5p, 6q, 8q, 18p, and 20q, mainly the specific regions 1p34, 2p24, 2q24-34, 3q22-ter, 7p12-22, 8q13-qter, 11p11.2, 11q13, 12p11.2, 13q21-34, 17q12, 20q12-13 and Xq27-28, may contain ESCC-related oncogenes; 1p, 3p, 4p, 8p, 9p13, 9q and 19p may contain ESCC-related tumor suppressor genes involved in the development and progression of esophageal cancer (Figure 1B).

Early genomic changes and imbalances associated with tumor staging

It has been known that ESCC arise through multi-step genetic and cytogenetic alterations. The time sequence of these alterations, however, remains to be identified. Thus studies that correlate chromosomal aberrations with stage and clinical outcome of prognostic significance are necessary to assist in the selection of patients for specific treatments.

An interesting and recently published study explored the application of M-FISH for early diagnosis and risk prediction of precursor lesions of ESCC in tumor and premalignant lesions in 113 patients (Yao *et al.* 2008). Elevated aneuploidy rates of chromosomes 3, 8, 10, 12, 17 and 20 were frequently found both in ESCC and in its precursor dysplastic lesions. These findings support the conclusion that a multi-target FISH assay investigating chromosomal aneuploidy in esophageal dysplastic sites may be useful to predict risk to ESCC.

The progression to advanced, metastatic stage is accompanied by genomic changes, as detected by mCGH analyses in lymph node metastasis of ESCC. Copy number gains were frequently

detected at 1q, 1p36.32, 3q, 5p, 8q23-qter, 11q13-14, 5p14-pter, 6p, 20q, 7p22.3, 7q, 2p, 12p, 19p13.3 and 20p and DNA amplifications were detected at 11q13, 2q12, 6p12-6q12, 7q21, 20q11.2 and 20p12. Losses were detected at 18q, 3p, 9p, 5q14-23, 4q, 13, and 11q22-qter (Qin *et al.*, 2005a; Wang *et al.*, 2006; Carneiro *et al.*, 2008; Qin *et al.*, 2008).

Gain of 3q, 5p, 1q, 11q13-14 and loss of 4 and 13q were all significantly correlated with pathologic staging, while gains of 8q, loss of 4p were linked to nodal metastasis and gains of 2p and loss of 4 and 11q14-qter were associated with distant organ metastasis (Qin *et al.*, 2004). It is likely that gain of 1q, 3q, 5p, , 11q13-14 and loss of 4 and 13q are the genetic aberrations critical for the development of esophageal carcinoma, whereas gains of 2p and 8q, and loss of 4 and , 11q14-qter are considered later events associated with tumor progression and are thought to confer metastatic potential to esophageal carcinoma. Furthermore, nodal and distant organ metastases involve different genes (Qin *et al.*, 2005b). The gains of 3q and 11q13 and losses of 3p, 4q, 5q14-23, 9p and 18q were detected in both early and advanced stage ESCC.

It was found that deletions of 4p and 13q12–q14 and gain of 5p were significantly correlated with pathologic staging. Losses of 8p22-pter and 9p also were found more frequently in patients with advanced disease. Gain of 8q24-qter was seen more frequently in patients with grade 3 tumors (Yen *et al.*, 2001). Shiomi *et al.* (2003) using mCGH, observed that gains of 3q, 8q, 11q13 and 14q were early events, while losses of 3p, 5q, 13q and 21q, and gains of 1p and Xq were later events in progression of individual tumors.

Gains at 6p and 20p, and losses at 10p and 10q are the most significant imbalances, as in primary carcinoma as in metastasis, suggesting the regions could have oncogenes and tumor suppressor genes (Qin *et al.*, 2005a). However, gain in 12p, and loss in 3p could be associated with poor relapse-free survival (Kwong *et al.*, 2004).

Searching for relevant oncogenes in ESCC

In 41 primary ESCC investigated by technology??(Fujita *et al.* 2003); (Xu *et al.*2007), numerous genes have been seen amplified, such as the cell cycle-regulator kinase *BTAK* (20q13.2-q13.3) in 9.8%, *E2F1* (20q11.2), plays a crucial role in the control of cell cycle and action of tumor suppressor proteins) in 7.3%, *NCOA3* (20q12) in 4.9%, and *DcR3* (20q13.3), which plays a regulatory role in apoptosis) in 4.9%. Xu *et al.* (2007) detected *NCOA3* overexpression and increased copy number in 46% and 13% of 221 ESCCs, respectively. *NCOA3* encodes a nuclear receptor coactivator that interacts with nuclear hormone receptors to enhance their transcriptional activator functions. Its overexpression was observed more frequently in late T stages than in earlier stages, but no significant association of expression of *NCOA3* and lymph node metastases was observed. These results suggest that overexpression of *NCOA3* caused by genomic gain or other molecular mechanisms might provide a selective advantage for the development and local invasion of certain subsets of ESCC.

To investigate allelic imbalance of chromosome 18q in ESCC, FISH analysis was performed and two of five resected ESCC samples from patients showed loss of one copy of chromosome 18q and 13 of 46 ESCC samples (28.3%) showed loss of almost the entire 18q (Ando *et al.*, 2007). The authors suggested that 18q loss may play an important role in the progression of ESCC.

Some studies have been focused on the identification of genetic targets for identifying deranged pathways and clinically applicable markers. Oncogene amplification was examined with DNA microarrays in 20 surgically resected ESCC and 57 oncogenes were detected as amplified. Alterations in DNA copy numbers detected by microarrays were compared to those obtained by mCGH. Amplification of 8 oncogenes (*CCND1*, *FGF3/ FGF4*, *EMS1*, *SAS*, *ERBB2*, *PDGFRA*, *MYC*, and *BCL2*)

was detected by DNA microarrays in 9 of 20 tumors. Although *ERBB2* was 23-fold higher than the control level in one case, the average magnitude of gene amplification was approximately 2 - to 4 - fold that of the control level. *EMS1*, *CCND1*, and *FGF3/FGF4*, which are all located on 11q13, were amplified in 7, 5, and 4 of 20 ESCC, respectively, and they were co-amplified in 3 tumors. *EMS1* has two roles: it regulates the interactions between components of adherens-type junctions and organize the cytoskeleton and cell adhesion structures of epithelia and carcinoma cells (National Center for Biotechnology Information, 2009). *FGF4/FGF3* broad the mitogenic, cell survival and oncogenic activity. A comparison of genome DNA microarrays and mCGH data revealed that although most amplified oncogenes were included in chromosomal regions for which DNA copy number gains were detected by mCGH, not all amplified genes on microarrays showed concomitant DNA copy number gains on mCGH, indicating that the resolution reached by the mCGH technique was limited. Microarrays of oncogenes are useful for the comprehensive identification of amplified oncogenes and for analysis of areas of specific amplification within chromosomal regions with DNA copy number increases detected by mCGH analysis (Arai *et al.*, 2003).

FISH assays revealed amplification of *PLK1* (polo-like kinase 1 (*Drosophila*)), an essential gene for the maintenance of genomic stability during mitosis (Feng *et al.*, 2009), suggesting that *PLK1* might be a useful prognostic marker. Investigation on 108 ESCCs and 9 ESCC cell lines showed that *MDS1* (myelodysplasia syndrome 1) and *PRKCI* (protein kinase C, iota), genes implicated in neoplastic transformation, were frequently gained and a positive correlation was found for *PRKCI* between amplification and tumor size, lymph node metastasis and clinical stage. *PRKCI* gene amplification was highly correlated with protein overexpression suggesting that gene amplification is one important mechanism involved in *PRKCI* overexpression (Yang *et al.*, 2008b).

Overexpression of epidermal growth factor receptor (*EGFR*, epidermal growth factor receptor) is observed in many cancers, sometimes accompanied by gene amplification. By FISH, *EGFR* gene was found amplified in ESCC (Sunpaweravong *et al.*, 2005; Hanawa *et al.*, 2006). Also, *EGFR* gene overrepresentation (balanced gene and chromosome 7 polysomy) and *HER-2* amplification were common events (Mimura *et al.*, 2005; Sunpaweravong *et al.*, 2005; Bizari *et al.*, 2006).

Cyclin D1 (*CCDN1*) is a cell-cycle regulator and an oncogene implicated in the pathogenesis of numerous tumor types. Amplification of the *CCDN1* gene occurs commonly in ESCC and it was detected by FISH (Sheyn *et al.*, 1997; Jin *et al.*, 2004; Manoel-Caetano *et al.*, 2004; Sunpaweravong *et al.*, 2005; Bizari *et al.*, 2006).

Correlation between the overexpression and amplification of oncogenes with survival has not been extensively investigated, however, the survival of patients with *BTAK* or *E2F1* amplification was significantly lower than that of patients without these abnormalities, suggesting that amplification of those genes is likely to lead to an increase in the number of malignant ESCC phenotypes and that aberrations can be expected to be useful as marker of poor prognosis (Fujita *et al.*, 2003).

Concluding Remarks

The molecular cytogenetic approaches have been useful to demonstrate the extensive genetic complexity in the different stages of ESCC, supporting the involvement of various key genes in the esophageal carcinogenesis. Most of the unbalanced chromosomal regions were similar in primary tumors and cell lines, confirming that cell lines are reliable models for investigation of molecular mechanisms in effect in patient specimens.

There were specific imbalanced patterns during tumor progression. While gains at 1q, 5p, 8q, 14q and losses at 4p, 13q and 18q are related to early stages of ESCC development; gains at 1p, 2p, 7p22.3, 8q, 8q24-qter, Xq and losses at 8p22-pter, 11q14-qter, 13q and 21q are associated with advanced stage. Gains at 3q and 11q13, and losses at 3p, 4q, 5q14-23, 9p, 18q were detected in both early and advanced stage of ESCC. These regions harbor genes relevant for essential cellular processes (e.g., regulation of transcription, signal transduction, cell proliferation and cell differentiation) that participate of central signaling cascades impacting cancer development. Some imbalances may indicate a poor prognostic marker such as losses at 4p, 9p, 11q and gains at 1p36-32, 5p, 7q, 19p13.3. Gene amplifications affected mostly oncogenes like *YES-1*, *TYMS*, *HEC*, *TGIF*, *NCOA-3*, *BTAK*, *DcR-3*, *E2F1*, *MYC*, *EGFR*, *EGR2*, *CCND1/ Cyclin D1*, *FGF3/FGF4*, *EMS1*, *SAS*, *ERBB2 (Her-2-neu)*, *PDGFR1*, *BCL2*, *MDS1* and *PRKCI*, while genomic losses lead to deletions of suppressor tumor genes *CDKN2A*, *MTAP*, *TP53*.

The identification of these critical genes in esophageal carcinogenesis will assist the development of individualized target therapy, which is expected to positively impact the clinical outcome of ESCC patients.

References

- Ando T, Ishiguro H, Kimura M, Mitsui A, Mori Y, Sugito N, Tomoda K, Mori R, Harada K, Katada T, Ogawa R, Fujii Y and Kuwabara Y (2007) Frequent loss of the long arm of chromosome 18 in esophageal squamous cell carcinoma. *Oncol Rep.* 17(5):1005-1011.
- Arai H, Ueno T, Tangoku A, Yoshino S, Abe T, Kawauchi S, Oga A, Furuya T, Oka M and Sasaki K (2003) Detection of amplified oncogenes

by genome DNA microarrays in human primary esophageal squamous cell carcinoma: comparison with conventional comparative genomic hybridization analysis. *Cancer Genet Cytogenet.* 146:16–21.

Bauman JG, Wiegant J, Borst P and van Duijn P (1980) A new method for fluorescence microscopical localization of specific DNA sequences by *in situ* hybridization of fluorochromelabelled RNA. *Exp Cell Res.* 128:485–490.

Bizari L, Borim AA, Leite KR, Gonçalves F de T, Cury PM, Tajara EH and Silva AE (2006) Alterations of the CCND1 and HER-2/neu (ERBB2) proteins in esophageal and gastric cancers. *Cancer Genet Cytogenet.* 165(1):41-50.

Carneiro A, Isinger A, Karlsson A, Johansson J, Jönsson G, Bendahl PO, Falkenback D, Halvarsson B and Nilbert M (2008) Prognostic Impact of Array-based Genomic Profiles in Esophageal Squamous Cell Cancer. *BMC Cancer* 8:98-107.

Crawford JM (2004) The Gastrointestinal Tract. In: Cotran RS, Kumar V and Collins T *Pathologic basis of disease.* 7th ed. Philadelphia: WB Saunders Company, pp. 775–787.

Daigo Y and Nakamura Y (2008) From cancer genomics to thoracic oncology: discovery of new biomarkers and therapeutic targets for lung and esophageal carcinoma. *Gen Thorac Cardiovasc Surg.* 56(2):43-53.

D'Amico TA (2006) Molecular biologic staging of esophageal cancer. *Thorac Surg Clin.* 16(4):31-327.

du Manoir S, Speicher MR, Joos S, Schröck E, Popp S, Döhner H, Kovacs G, Robert-Nicoud M, Lichter P, Cremer T (1993) Detection of complete

and partial chromosome gains and losses by comparative genomic *in situ* hybridization. Hum Genet. 90:590–610.

Feng YB, Lin DC, Shi ZZ, Wang XC, Shen XM, Zhang Y, Du XL, Luo ML, Xu X, Han YL *et al.* (2009) Overexpression of PLK1 is associated with poor survival by inhibiting apoptosis via enhancement of survivin level in esophageal squamous cell carcinoma. Int J Cancer. 124(3):578-588.

Fiegler H, Carr P, Douglas EJ, Burford DC, Hunt S, Scott CE, Smith J, Vetrie D, Gorman P, Tomlinson IP *et al.* (2003) DNA microarrays for comparative genomic hybridization based on DOP-PCR amplification of BAC and PAC clones. Genes Chromosomes Cancer 36:361– 374.

Fujita Y, Sakakura C, Shimomura K, Nakanishi M, Yasuoka R, Aragane H, Hagiwara A, Abe T, Inazawa J and Yamagishi H (2003) Chromosome arm 20q gains and other genomic alterations in esophageal squamous cell carcinoma, as analyzed by comparative genomic hybridization and fluorescence in situ hybridization. Hepatogastroenterology 50(54):1857-63.
Hanawa M, Suzuki S, Dobashi Y, Yamane T, Kono K, Enomoto N and Ooi A (2006) EGFR protein overexpression and gene amplification in squamous cell carcinomas of the esophagus. Int J Cancer. 118(5):1173-1180.

Hsia JY, Chen CY, Chen JTA, Hsu CP, Shai SE, Yang SS, Chuang CY, Wang PY and Miaw J (2003) Prognostic significance of caspase-3 expression in primary resected esophageal squamous cell carcinoma. EJSO. 2944±48.

Huang XP, Rong TH, Wang JY, Tang YQ, Li BJ, Xu DR, Zhao MQ, Zhang LJ, Fang Y, Su XD *et al.* (2006) Negative implication of C-MYC as an

amplification target in esophageal cancer. *Cancer Genet Cytogenet.* 165(1):20-24.

Jin Y, Jin C, Law S, Chu KM, Zhang H, Strombeck B, Yuen AP and Kwong YL (2004) Cytogenetic and fluorescence in situ hybridization characterization of clonal chromosomal aberrations and CCND1 amplification in esophageal carcinomas. *Cancer Genet Cytogenet.* 148(1):21-28.

Kallioniemi A, Kallioniemi OP, Sudar D, Rutovitz D, Gray JW, Waldman F and Pinkel D (1992) Comparative genome hybridization for molecular cytogenetics analysis of solid tumors. *Science* 258 (5083):818-821.

Kamitani S, Sugihara H, Shiomi H, Tani T and Hattori T (2002) Intratumoral regional variations in copy number of the chromosomal part revealed by microdissection and combined ploidy and comparative genomic hybridization analyses in esophageal squamous cell carcinoma. *Cancer Genet Cytogenet.* 132:30–35

Khushalani N (2008) Cancer of the esophagus and stomach. *Mayo Clin Proc.* 83(6):712-722.

Knudson AG (1985) Hereditary Cancer, oncogenes, and anti-oncogenes. *Cancer Res.* 45:1437–1443.

Koch WM, Boyle JO, Mao L, Hankim J, Hruban RH and Sidransky D (1994) TP53 gene mutations as markers of tumor spread in synchronous oral cancer. *Arch. Otolaryngol Head Neck Surg.* 120:943–947.

Kuwano H, Kato H, Miyazaki T, Fukuchi M, Masuda N, Nakajima M, Fukai Y, Sohda M, Kimura H and Faried A (2005) Genetic alterations in esophageal cancer. *Surg Today* 35:7-18.

Kwong D, Lam A, Guan X, Law S, Tai A, Wong J and Sham J (2004) Chromosomal aberrations in esophageal squamous cell carcinoma among Chinese: gain of 12p predicts poor prognosis after surgery. *Hum Pathol.* 35(3):309-316.

Kwong KF (2005) Molecular Biology of Esophageal Cancer in the Genomics Era. *Surg Clin N Am.* 85:539–553.

Lam AK (2000) Molecular biology of esophageal squamous cell carcinoma. *Crit Rev Oncol Hematol.* 33:71–90.

Langer PR, Waldrop AA and Ward DC (1981) Enzymatic synthesis of biotin-labeled polynucleotides: novel nucleic acid affinity probes. *Proc. Natl Acad. Sci. USA* 78:6633–6637.

Makhija S, Sit A, Edwards R, Aufman K, Weiss H, Kanbour-Shakir A, Gooding W, D'angelo G, Ferrell R, Raja S *et al.* (2003) Identification of genetic alteration related to chemoreistence in ephitelial ovarian cancer. *Gynecol Oncol.* 90:3-9.

Mandard AM, Hainaut P and Hollstein M (2000) Genetic steps in the development of squamous cell carcinoma of the esophagus. *Mutat Res.* 462:335-342.

Manoel-Caetano F da S, Borim AA, Caetano A, Cury PM, Silva AE (2004) Cytogenetic alterations in chagasic achalasia compared to esophageal carcinoma. *Cancer Genet Cytogenet.* 149(1):17-22.

Mayama T, Fukushima S, Shineha R, Nishihira T, Satomi S, Horii A (2000) Frequent loss of copy number on the long arm of chromosome 21 in human esophageal squamous cell carcinoma. *Int J Oncol.* 17(2):245-252

McCabe ML and Dlamini Z (2005) The molecular mechanisms of oesophageal cancer. *Int Immunopharmacol.* 5:1113–1130.

Mimura K, Kono K, Hanawa M, Mitsui F, Sugai H, Miyagawa N, Ooi A and Fujii H (2005) Frequencies of HER-2/neu expression and gene amplification in patients with oesophageal squamous cell carcinoma. *Br J Cancer* 92(7):1253-60.

Muñoz N (1997) Le dépistage du cancer de l'oesophage est-il réalisable? *Semaines des Hôpitaux de Paris* 73:633-635.

Nakakuki K, Imoto I, Pimkhaokham A, Fukuda Y, Shimada Y, Imamura M, Amagasa T and Inazawa J (2002) Novel targets for the 18p11.3 amplification frequently observed in esophageal squamous cell carcinoma. *Carcinogenesis* 23 (1):19-24.

Pack SD, Karkera JD, Zhuang Z, Pak ED, Balan KV, Hwu P, Park WS, Pham T, Ault DO, Glaser M *et al.* (1999) Molecular cytogenetic fingerprinting of esophageal squamous cell carcinoma by comparative genomic hybridization reveals a consistent pattern of chromosomal alterations. *Genes Chromosomes Cancer* 25(2):160-168.

Pelengaris S and Khan M (2003) The many faces of c-MYC. *Arch Biochem Biophys.* 416(2):129-136.

Pimkhaokham A, Shimada Y, Fukuda Y, Kurihara N, Imoto I, Yang ZQ, Imamura M, Nakamura Y, Amagasa T and Inazawa J (2000) Nonrandom Chromosomal Imbalances in Esophageal Squamous Cell Carcinoma Cell Lines: Possible Involvement of the *ATF3* and *CENPF* Genes in the 1q32 Amplico Jpn J Cancer Res. 91:1126–1133.

Pinkel D, Segreaves R, Sudar D, Clark S, Poole I, Kowbel D, Collins C, Kuo WL, Chen C, Zhai Y *et al.* (1998) High resolution analysis of DNA copy number variations using comparative genomic hybridization to microarrays. Nat Genet. 20:207–211

Qin YR, Wang LD, Kwong D, Guan XY, Zhuang ZH, Fan ZM, An JY and Tsao G (2004) Comparative genomic hybridization of esophageal squamous cell carcinoma and gastric cardia adenocarcinoma in high-incidence region of esophageal carcinoma. Linzhou Henan. Zhonghua Yi Xue Yi Chuan Xue Za Zhi 21(6):625-8

Qin YR, Wang LD, Dora K, Guan XY, Zhuang ZH, Fan ZM, Deng W and Cao SH (2005a) Genomic changes in primary lesion and lymph node metastases of esophageal squamous cell carcinoma. Ai Zheng 24(9):1048-1053.

Qin YR, Wang LD, Kwong D, Gao SS, Guan XY, Zhuang ZH, Fan ZM, Deng W and Hu L (2005b) Comparative genomic hybridization: the profile of chromosomal imbalances in esophageal squamous cell carcinoma. Zhonghua Bing Li Xue Za Zhi 34(2):80-83.

Qin YR, Wang LD, Fan ZM, Kwong D and Guan XY (2008) Comparative genomic hybridization analysis of genetic aberrations associated with development of esophageal squamous cell carcinoma in Henan, China. World J Gastroenterol. 14(12):1828-1835.

Schröck E, du Manoir S, Veldman T, Schoell B, Wienberg J, Ferguson-Smith MA, Ning Y, Ledbetter DH, Bar-Am I, Soenksen D *et al.* (1996) Multicolor spectral karyotyping of human chromosomes. *Science* 273:494–497.

Schröck E, Padilla-Nash H (2000) Spectral karyotyping and multicolor fluorescence in situ hybridization reveal new tumor-specific chromosomal aberrations. *Semin Hematol.* 37(4):334-47.

Sherry ST, Ward MH, Kholodov M, Baker J, Phan L, Smigielski EM, Sirotkin K (2001) dbSNP: the NCBI database of genetic variation. *Nucleic Acids Res.* 29:308–311.

Sheyn I, Noffsinger AE, Heffelfinger S, Davis B, Miller MA and Fenoglio-Preiser CM (1997) Amplification and expression of the cyclin D1 gene in anal and esophageal squamous cell carcinomas. *Hum Pathol.* 28(3):270-276.

Shimada Y, Imamura M, Wagata T, Yamaguchi N and Tobe T (1992) Characterization of 21 newly established esophageal cancer cell lines. *Cancer* 69:277-284.

Shinomiya T, Mori T, Ariyama Y, Sakabe T, Fukuda Y, Murakami Y, Nakamura Y and Inazawa J (1999) Comparative genomic hybridization of squamous cell carcinoma of the esophagus: The possible involvement of the *DP1* gene in the 13q34 amplicon. *Genes Chromosomes Cancer* 24:337–344.

Shiomi H, Sugihara H, Kamitani S, Tokugawa T, Tsubosa Y, Okada K, Tamura H, Tani T, Kodama M and Hattori T (2003) Cytogenetic

heterogeneity and progression of esophageal squamous cell carcinoma. *Cancer Genet Cytogenet.* 147(1):50-61.

Snijders AM, Nowak N, Segraves R, Blackwood S, Brown N, Conroy J, Hamilton G, Hindle AK, Kimura K, Law S *et al.* (2001) Assembly of microarrays for genome-wide measurement of DNA copy number. *Nat Genet.* 29(3):263-264.

Solinas-Toldo S, Lampel S, Stilgenbauer S, Nickolenko J, Benner A, Döhner H, Cremer T, Lichter P (1997) Matrix-based comparative genomic hybridization: biochips to screen for genomic imbalances. *Genes Chromosomes Cancer* 20: 399–407.

Somers KD and Schechter GL (1992) Genetic alterations in head and neck cancer. *Mol Biol Genetics* 25(5):1065 – 1071.

Speicher MR, Ballard SG and Ward DC (1996) Karyotyping human chromosomes by combinatorial multi-fluor FISH. *Nat Genet.* 12:368–375.

Speicher MR and Carter NP (2005) The new cytogenetics: blurring the boundaries with molecular biology. *Nature* 6:782 – 792.

Su M, Chin SF, Li XY, Edwards P, Caldas C and Fitzgerald RC (2006) Comparative genomic hybridization of esophageal adenocarcinoma and squamous cell carcinoma cell lines. *Dis Esophagus* 19(1):10-14

Sugimoto T, Arai M, Shimada H, Hata A and Seki N (2007) Integrated analysis of expression and genome alteration reveals putative amplified target genes in esophageal cancer. *Oncol Rep.* 18:465-472.

Sunpaweravong P, Sunpaweravong S, Puttawibul P, Mitarnun W, Zeng C, Barón AE, Franklin W, Said S and Varella-Garcia M (2005) Epidermal growth factor receptor and cyclin D1 are independently amplified and overexpressed in esophageal squamous cell carcinoma. *J Cancer Res Clin Oncol.* 131(2):111-119.

Tada K, Oka M, Hayashi H, Tangoku A, Oga A and Sasaki K (2000) Cytogenetic analysis of esophageal squamous cell carcinoma cell lines by comparative genomic hybridization: relationship of cytogenetic aberrations to in vitro cell growth. *Cancer Genet Cytogenet.* 117(2):108-112.

Wang LD, Qin YR, Fan ZM, Kwong D, Guan XY, Tsao GS, Sham J, Li JL and Feng XS (2006) Comparative genomic hybridization: comparison between esophageal squamous cell carcinoma and gastric cardia adenocarcinoma from a high-incidence area for both cancers in Henan, northern China. *Dis Esophagus* 19(6):459-67.

Wu YP, Yang YL, Yang GZ, Wang XY, Luo ML, Zhang Y, Feng YB, Xu X, Han YL, Cai Y *et al.* (2006) Identification of chromosome aberrations in esophageal cancer cell line KYSE180 by multicolor fluorescence in situ hybridization. *Cancer Genet Cytogenet.* 170(2):102-107.

Xu FP, Xie D, Wen JM, Wu HX, Liu YD, Bi J, Lv ZL, Zeng YX and Guan XY (2007) *SRC-3/AIB1* protein and gene amplification levels in human esophageal squamous cell carcinomas. *Cancer Lett.* 245 (1-2):69-74.

Xue LY, Hu N, Song YM, Zou SM, Shou JZ, Qian LX, Ren LQ, Lin DM, Tong T, He ZG *et al.* (2006) Tissue microarray analysis reveals a tight correlation between protein expression pattern and progression of esophageal squamous cell carcinoma. *BMC Cancer* 6:296-311.

Yang YL, Chu JY, Wu Y, Luo ML, Xu X, Han YL, Cai Y, Zhan Q and Wang M (2008a) Chromosome analysis of esophageal squamous cell carcinoma cell line KYSE 410-4 by repetitive multicolor fluorescence in situ hybridization. *J Genet Genomics* 35(1):11-16.

Yang YL, Chu JY, Luo ML, Wu YP, Zhang Y, Feng YB, Shi ZZ, Xu X, Han YL, Cai Y *et al.* (2008b) Amplification of PRKCI, located in 3q26, is associated with lymph node metastasis in esophageal squamous cell carcinoma. *Genes Chromosomes Cancer* 47(2):127-36.

Yao HQ, He S, Wu YP, Wang XC, Han YL, Xu X, Cai Y, Wang GQ and Wang MR (2008) Application of Multicolor Fluorescence In situ Hybridization to Early Diagnosis of Esophageal Squamous Cell Carcinoma. *Ai Zheng* (11):1137-43.

Yen CC, Chen YJ, Chen JT, Hsia JY, Chen PM, Liu JH, Fan FS, Chiou TJ, Wang WS, Lin CH (2001) Comparative genomic hybridization of esophageal squamous cell carcinoma: correlations between chromosomal aberrations and disease progression/prognosis. *Cancer* 92(11):2769-2777.

Yen CC, Chen YJ, Lu KH, Hsia JY, Chen JT, Hu CP, Chen PM, Liu JH, Chiou TJ, Wang WS *et al.* (2003) Genotypic analysis of esophageal squamous cell carcinoma by molecular cytogenetics and real-time quantitative polymerase chain reaction. *Int J Oncol* 23: 871-881.

Internet Resources Section

AMERICAN CANCER SOCIETY, <http://www.cancer.org> (December 8th, 2008)

GENOME, NATIONAL HUMAN GENOME RESEARCH INSTITUTE,
[http://www.genome.gov/Pages/Hyperion/DIR/VIP/Glossary/Illustration/fish.cfm?key=fluorescence%20in%20situ%20hybridization%20\(FISH\)](http://www.genome.gov/Pages/Hyperion/DIR/VIP/Glossary/Illustration/fish.cfm?key=fluorescence%20in%20situ%20hybridization%20(FISH)) (January 20th, 2009)

INSTITUTO NACIONAL DO CÂNCER (INCA), <http://www.inca.org.br>
(December 8th, 2008)

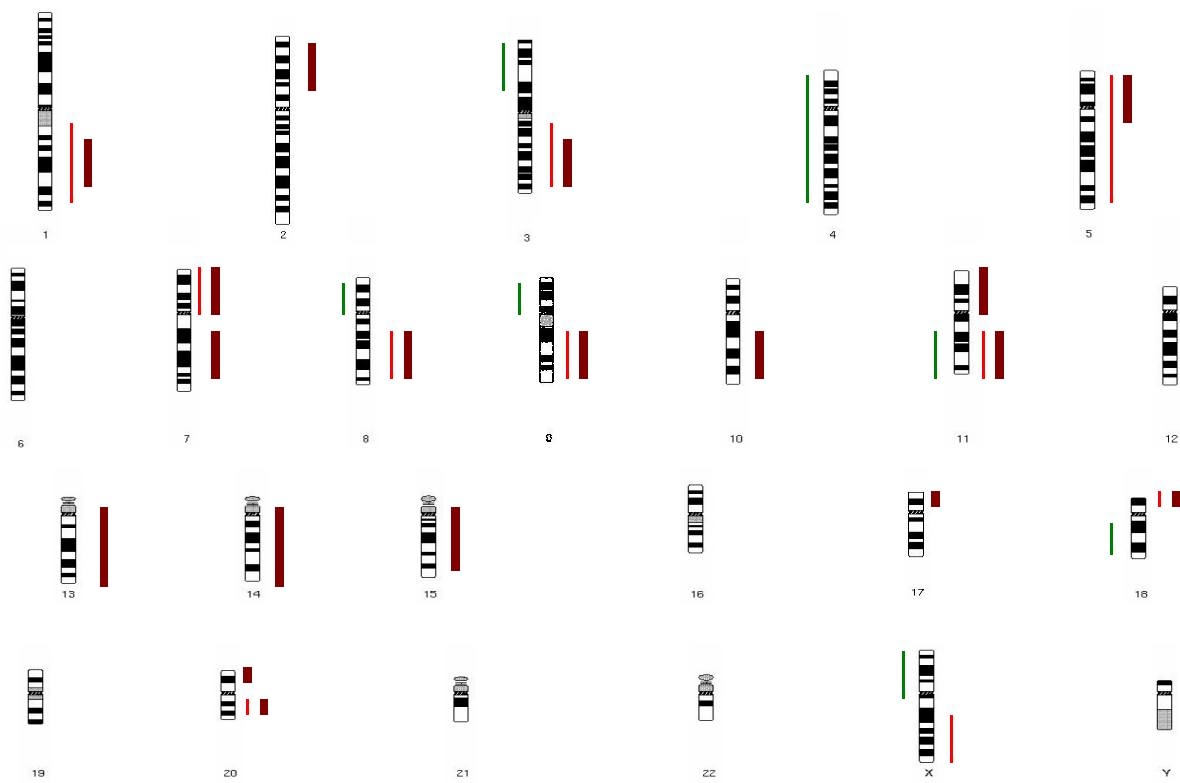
MEDICAL GENETICS LABORATORIES,
<http://www.bcm.edu/geneticlabs/tests/cyto/105Kcma.html> (December 22nd, 2008)

NATIONAL CENTER FOR BIOTECHNOLOGY INFORMATION,
<http://www.ncbi.nlm.nih.gov/sites/entrez>, (March 5th, 2009)

Legend of figures:

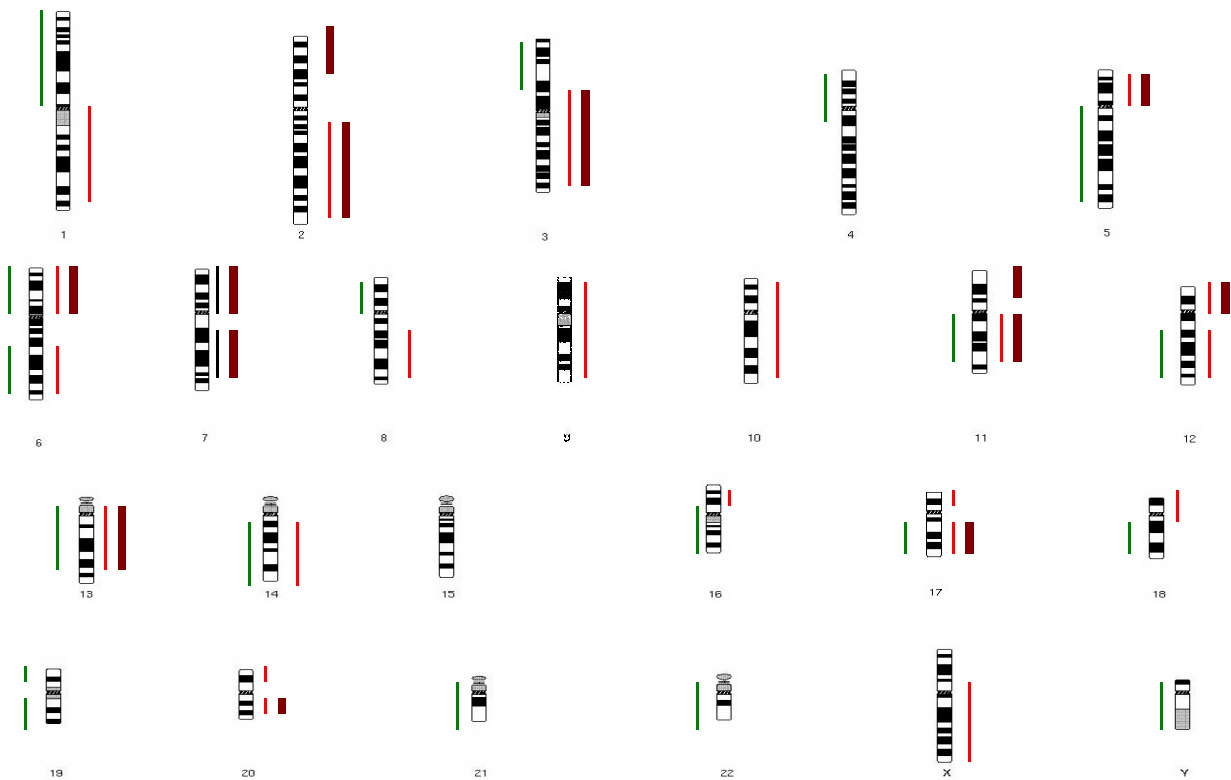
Figure 1. Summary of copy number alterations. in esophageal squamous cell carcinomas analyzed by comparative genomic hybridization. Chromosomal regions of gain are represented in red on the right side of the chromosome ideograms, the amplification regions are represented by the larger dark red line on the right side of the chromosome ideograms and regions of loss are represented in green on the left side. A. cell lines; B. tumor

A.



— Loss
— Gain
— Amplification

B.



— Loss
— Gain
— Amplification

Capítulo II

CAPÍTULO II: p53, p16 and Fhit Proteins Expressions in Chronic Esophagitis and Chagas Disease

Authors: Marilanda Ferreira Bellini¹; Kátia Ramos Moreira Leite², Patrícia Maluf Cury³ and Ana Elizabete Silva¹

Authors Addresses:

¹ Department of Biology, UNESP, São Paulo State University, Campus São José do Rio Preto, SP;

² Laboratory of Surgery and Molecular Pathology, Hospital Sírio Libanês, São Paulo, SP;

³ Department of Pathology, FAMERP – Medicine School, São José do Rio Preto, SP, Brazil

Correspondence to: Ana Elizabete Silva, IBILCE/UNESP – Departamento de Biologia, Rua Cristóvão Colombo, 2265, São José do Rio Preto, SP, Brazil - CEP. 15054-000.

Tel:+ 551732212384, e-mail: anabete@ibilce.unesp.br

Key Words: p53, p16, Fhit protein, esophagitis, megaesophagus, esophageal carcinoma.

Running Title: Bellini *et al.*: p53, p16 and Fhit Protein Expressions in Esophageal Lesions

Supported by Brazilian Agencies FAPESP and CNPq

Artigo original publicado na revista

Anticancer Research (ISSN:1791-7530)

28(6A):3793-3800, 2008.

Resumo

Introdução: Alguns modelos sugerem que o desenvolvimento do carcinoma esofágico é resultante de uma sequência de alterações, que envolvem esofagite, atrofia, displasia, carcinoma *in situ* e carcinoma invasivo. Enquanto diversas alterações genéticas têm sido descritas em carcinoma de esôfago, os estudos em lesões benignas com potencial pré-canceroso ainda são escassos. *Material e Métodos:* Foi realizada imuno-histoquímica para as proteínas p53, p16 e Fhit em mucosa esofágica de pacientes com Doença de Chagas (CD), megaesôfago chagásico (CM), esofagite crônica (CE), carcinoma de células escamosas de esôfago (ESCC) e em mucosa normal (NM). *Resultados:* A proporção de casos positivos para a proteína p53 aumentou progressivamente de acordo com a severidade da lesão, CD (7.7%), CM (26.1%), CE (52.2%) and ESCC (100%). Entretanto, as proteínas p16 e Fhit não mostraram diferenças estatisticamente significantes entre os grupos. *Conclusão:* O presente estudo mostra o envolvimento da proteína p53 super expressa nos processos iniciais da carcinogênese esofágica, sugerindo sua utilização como marcador em lesões precursoras, inversamente, perdas na expressão de Fhit e p16 não foram significantes

p53, p16 and Fhit Proteins Expressions in Chronic Esophagitis and Chagas Disease

MARILANDA FERREIRA BELLINI¹, KÁTIA RAMOS MOREIRA LEITE²,
PATRÍCIA MALUF CURY³ and ANA ELIZABETE SILVA¹

¹Department of Biology, UNESP, São Paulo State University, Campus São José do Rio Preto, SP;

²Laboratory of Surgery and Molecular Pathology, Hospital Sírio Libanês, São Paulo, SP;

³Department of Pathology, FAMERP – Medical School, São José do Rio Preto, SP, Brazil

Abstract. *Background: Models have suggested esophageal carcinogenesis can result from the alteration of sequences, leading to esophagitis, atrophy, dysplasia, carcinoma in situ and invasive carcinoma. While numerous genetic alterations have been reported in esophageal carcinogenesis, studies of benign lesions with precancerous potential are scarce. Materials and Methods: Immunohistochemistry was performed for p53, p16 and Fhit proteins in the esophageal mucosa from patients with Chagas disease (CD), chagasic megaesophagus (CM), chronic esophagitis (CE), esophageal squamous cell carcinoma (ESCC) and in normal mucosa (NM). Results: The proportion of p53-positive cases increased progressively according to the severity of the pathology CD (7.7%), CM (26.1%), CE (52.2%) and ESCC (100%). However, p16 and Fhit did not show any statistically significant differences among the groups. Conclusion: p53 overexpression is involved in the initial steps of esophageal carcinogenesis, supporting further evaluation of its utility as a marker in precursor lesions, conversely, losses of Fhit and p16 expression may not be significant.*

The development of human esophageal cancer is a multi-step, progressive process. An early indicator of this process is increased proliferation of esophageal epithelial cells morphologically including basal cell hyperplasia, dysplasia, carcinoma *in situ* and invasive carcinoma (1). Patients with histological evidence of esophagitis, a promoter of the inflammatory process in the esophageal epithelium, may

have a moderately increased risk of esophageal squamous cell carcinoma (ESCC) (1-3). Similarly, one of the serious late consequences of chagasic megaesophagus (CM) (esophagus dilation) due to achalasia in Chagas disease (CD) patients is the increased risk, around 3% to 8%, of these patients developing esophageal carcinoma (4, 5).

The process of tumorigenesis at the cellular level is related to disorders of cell proliferation control, differentiation and apoptosis (6). Genetic changes associated with ESCC development include alterations in the tumor suppressor genes *TP53* (17p13.1), *CDKN2A/P16* (9p21) and *FHIT* (3p14.2), and the oncogenes *c-MYC* (8q24), *CCND1* (11q13), *EGFR* (7p11-15), *ERBB2* (17q11.2-q12) and *HGF* (7q21.1), such as the regulation of their respective protein expressions (1, 7, 8-20).

While numerous genetic and epigenetic alterations have been reported in the initial and advanced steps of esophageal carcinogenesis, studies are scarce regarding benign lesions with precancerous potential (5, 21-23). The aim of this study was to evaluate the levels of p53, p16 and Fhit protein expression in precursor lesions, such as CM and chronic esophagitis (CE) in cancer-free patients, to determine the relationship between these lesions and tumor development.

Materials and Methods

Samples. A total of 80 specimens of paraffin-embedded esophageal tissue were obtained from patients who had undergone middle and distal esophageal biopsies, before any chemo- or radiotherapy induction, at the Pathology Section, Hospital de Base (São José do Rio Preto, SP, Brazil). Twenty-three specimens were obtained from CD patients with CM, 13 from patients with CD but without CM, 23 from CE patients and 11 from ESCC. Both patients with CD and CE were esophageal cancer free. Esophageal mucosa from 10 healthy individuals were obtained and diagnosed as histologically normal (NM). The study was approved by the Brazilian National Research Ethics Committee (CONEP).

Immunohistochemical assay. Consecutive 4 µm-thick sections were cut from each trimmed paraffin block and mounted on glass slides

Supported by Brazilian Agencies FAPESP and CNPq.

Correspondence to: Ana Elizabete Silva, IBILCE/UNESP – Departamento de Biologia, Rua Cristóvão Colombo, 2265, São José do Rio Preto, SP, Brazil - CEP. 15054-000. Tel: +55 1732212384, e-mail: anabete@ibilce.unesp.br

Key Words: p53, p16, Fhit protein, esophagitis, megaesophagus, esophageal carcinoma.

pre-treated with 3-aminopropyl-triethoxysilane/acetone solution. In brief, following deparaffinization, the sections were re-hydrated, treated with citrate buffer at 96°C for 30 min and treated with 3% H₂O₂ in methanol (v/v) for 30 min to block endogenous peroxidases. The sections were then incubated for 16 hours at 4°C with specific antibodies: anti-p53 (clone DO-7, 1:200; Novocastra, Newcastle, UK), anti-p16 (clone 6H12, 1:50; Novocastra) or anti-Fhit (clone SPM472, 1:200; Abcam, Cambridge, USA). The slides were incubated with biotinylated secondary antibody, then with streptavidin-biotin peroxidase, following the manufacturer's instructions (Super ABC Universal Kit, Easy Path; Erviegas, São Paulo, Brazil to anti-p53; Dako Cytomation Kit, Strept ABComplex/HRP; Dako, Glostrup, Denmark to anti-p16 and anti-Fhit). The immunostain was visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB) containing 0.005% H₂O₂ and counterstained with hematoxylin. Negative controls were established by replacing the primary antibody with buffer solution. Breast carcinoma, anterior pituitary and normal breast tissues were used as positive controls for p53, p16 and Fhit antibodies, respectively.

For p53 protein, approximately 500 cells were counted for each sample. For p16 and Fhit proteins, all the prepared tissue was examined for each sample. All the analyses were conducted under light microscopy (×400 magnification). The index of positivity for p53 protein (p53+) was defined as >3% of cells showing strong brown nuclear staining (5 times the maximum value observed in NM).

Immunostaining for p16 (brown nuclear and cytoplasmic staining) and Fhit (brown cytoplasmic staining) proteins was graded by intensity of staining as negative *i.e.* (–) absence of brown staining, or (+) weakly stained; or as positive *i.e.* (++) moderately stained or (+++) strongly stained as described previously (9, 24, 25). Positive scores (++) corresponded to normal protein expression, while negative immunostaining (–/+) was associated with loss of protein expression. Areas that were poorly preserved, crushed, cauterized, folded, or retracted were specifically avoided.

Statistical analysis. Descriptive statistics, Kruskal-Wallis test with Dunn post-test, Fisher's exact test and paired Student *t*-test were used to determined statistical significance. The statistical analysis was carried out with GraphPad Instat 3 computer software (26). The level of significance was set as $p < 0.05$. The Kappa test was used to examine the agreement among the three proteins (altered or normal expression) in each patient in the groups evaluated. It was performed using an online tool (27).

Results

The demographic and clinicopathological data for all the groups are shown in Table I. Kruskal-Wallis test with Dunn post-test among the characteristics in each group indicated no significantly statistical difference only between gender relations. Table I also shows the immunohistochemical and statistical analysis for the proteins p53, p16 and Fhit.

Positive immunostaining for p53 protein was observed in a few cells in the normal mucosa group, which was interpreted as expression of wild-type p53 protein. A progressive increase of p53 protein expression was observed as follows: 7.7% (1/13) of CD, 26.1% (6/23) of CM, 52.2% (12/23) of CE and 100% (11/11) of ESCC (Figure 1 Aa-e).

Statistically significant differences were observed using the test Kruskal-Wallis test with Dunn post-test comparing all groups together, between each group and the carcinoma group, and comparing each group with the normal mucosa group. The immunohistochemical stain for p16 (Figure 1 Ba-e), and Fhit (Figure 1 Ca-e) proteins showed focal and/or diffuse distribution on the basal lamina on the tissue surface for both proteins. The establishment of statistical differences between proteins and groups were performed by Fisher's exact test and indicated that only the CE group showed significant statistical difference ($p = 0.0214$) in comparison with the normal mucosa for p16 protein. Despite the progressive loss of expression of p16 protein, no statistical difference was observed among the groups. But while in the NM, CD and CM groups immunostaining was diffuse, in CE and ESCC it was mainly cytoplasmic and focal. The absence of Fhit protein stain was not observed and the immunostaining was diffuse, ranging from weak to strongly stained. However, statistical significance was not found for Fhit expression between the studied groups.

There was no agreement in simultaneous altered expression of the three proteins (Agreement Analysis by Kappa) in each studied group, but associations between protein expressions, comparing two by two, were found (p53 and p16; p53 and Fhit; p16 and Fhit) by Fisher's exact test (Table II). Moreover, no significant associations were observed between altered expression of p53, p16 and Fhit proteins and demographic and clinicopathological parameters such as age, gender, smoking and or (data not shown) in the different groups.

Discussion

The age difference between the groups was highly significant. The age of patients with CD, CM and ESCC was greater than those with NM and CE, corroborating the finding that patients with CD can be asymptomatic for many years up to late diagnosis (28). Megaesophagus affects individuals between the second and fourth decades of life and the diagnosis of carcinoma is hampered by a number of factors that mask its symptoms (21, 28). CE is only differentiated from ESCC when smoking and alcohol habits are compared, in both subgroups, indicating that the esophagitis can be caused by several factors (28), independent of alcoholism and or smoking habit. However, associations between these parameters and altered expression of p53, p16 and Fhit proteins were not observed in the studied groups.

In the present study, an increased expression of p53 protein was progressively observed according to the severity of the lesion (CM, CE and ESCC). The strong and diffuse nuclear staining in the ESCC group probably arose from the high expression of mutant p53 protein, whereas in CE (with

Table I. Demographic and clinicopathological distribution, immunohistochemical and statistical analysis for the proteins p53, p16 and Fhit in esophageal mucosa.

Group	N	Age (years)	Gender		Smoking		Alcoholism		p53 +		p16 (−/+)		Fhit (−/+)		
		μ±SE Range	Male	Female	Yes	No	Yes	No	μ±SE Range	(n/N)	(%)	(n/N)	(%)	(n/N)	(%)
NM	10	42.4±4.81 26-67 ^a	(3/10) 30%	(7/10) 70% ^a	(3/10) 30%	(7/10) 70% ^{a,b}	(4/10) 40%	(6/10) 60% ^{a,b}	0.1±0.07 ^a 0-0.6	0/10	0 ^a	6/10	60 ^a	1/10	10 ^{ns}
CD	13	61.3±2.85 45-79 ^{b,c}	(4/13) 30.8%	(9/13) 70.2% ^a	(5/13) 38.5%	(8/13) 61.5% ^{a,b}	(4/13) 30.8%	(9/13) 69.2% ^{a,b}	1.1±0.28 ^{a,b} 0-3.6	(1/13)	7.7 ^a	10/13	76.9 ^{a,b}	1/13	7.7 ^{ns}
CM	23	64.3±2.08 57-83 ^c	(13/23) 56.5%	(10/23) 43.5% ^a	(13/23) 56.5%	(10/23) 43.5% ^{a,b}	(8/23) 34.8%	(15/23) 65.2% ^{a,b}	4.4±1.66 ^{a,b} 0-30.4	(6/23)	26.1 ^b	17/23	73.9 ^{a,b}	2/23	8.7 ^{ns}
CE	23	50.5±1.99 40-70 ^{a,b}	(11/23) 47.8%	(12/23) 52.2% ^a	(4/23) 17.4%	(19/23) 82.7 ^a	(1/23) 4.3%	(22/23) 95.7% ^a	13.8±2.81 ^b 0-42	(12/23)	52.2 ^b	22/23	95.7 ^b	8/23	34.8 ^{ns}
ESCC	11	56.5±2.68 42-69 ^{a,b,c}	(10/11) 90.9%	(1/11) 9.1% ^a	(9/11) 81.8%	(2/11) 18.2% ^b	(8/11) 72.7%	(3/11) 27.3% ^b	73.9±6.62 ^c 25.6-96	(11/11)	100 ^c	10/11	90.9 ^{a,b}	2/11	18.2 ^{ns}
Statistical analysis		<i>p</i> <0.0001 *	<i>p</i> =0.482 ns Kruskal-Wallis Test with Dunn post-test (<i>p</i> <0.05)		<i>p</i> =0.0062 *		<i>p</i> =0.0021 *		<i>p</i> <0.0001	Fisher's exact test (<i>p</i> <0.05)					

NM, normal mucosa; CD, Chagas disease patients without megaesophagus; CM, chagasic megaesophagus; CE, chronic esophagitis; ESCC, esophageal squamous cell carcinoma; N, number of specimens; μ , average; SE, standard error. Immunostain: p53+, immunostain $>3\%$; p16 and Fhit, (-/+) absent/ weak; n/N, number of specimens with protein expression alteration /total of specimens. Statistical analysis: *statistical difference; ^{a,b,c}groups with different letters are statistically different for the same variable; ^{ns}no statistical difference.

diffuse staining) and CM (strong and focal staining), it was not possible to indicate p53 as mutated protein. It may also have been due to the expression of wild-type p53 that accumulates in the cells as a consequence of the physiological and inflammatory processes in the esophageal epithelium.

Fagundes *et al.* (11) also detected a progressive increase of p53 expression from normal esophageal mucosa (12%), through moderate esophagitis (22%), severe esophagitis (33%), dysplasia (36%) to carcinoma (100%), most often in smokers and alcoholics. Thus they suggested that patients with CE and p53 protein overexpression have an increased risk of esophageal cancer.

Recently, p53 expression was examined by immunohistochemistry in patients with esophageal carcinoma and achalasia in regions of non-malignant epithelia, showing esophagitis and or dysplasia. The authors observed increased expression of p53 in tumors, but not in non-neoplastic epithelia, such as esophagitis and dysplasia. However, histological changes were observed in the achalasia epithelia, from esophagitis due to the stagnation of food, leading to high epithelial proliferation and increased expression of mutant p53 (29). Chagasic megaesophagus patients often have CE due to esophageal stasis that has extended the

esophagus, increasing the risk of bacterial proliferation and damage to the mucosa (21, 29, 30). These bacteria belong to the normal microflora of the mouth and oropharynx and create an environment for anaerobic microorganisms capable of reducing nitrate into nitrite, producing carcinogens which may trigger esophageal carcinogenesis (30).

In the present study, 70% CM patients also had CE, of whom 5/6 had altered p53 expression and soft to severe CE. Therefore, the altered expression of p53 in the CM (6/23) and in CE (12/23) patients may have occurred in response to the inflammatory process. In addition, a moderately increased risk of developing ESCC was reported in CE patients, suggesting that CE is the precursor lesion for squamous cell carcinoma (3).

Genetic alterations in dysplasia of esophageal carcinoma has shown p53 immunoreactivity ranging from 40% in moderate dysplasia to 48% in carcinoma, indicating an increase in p53 expression in dysplasia compared with both normal epithelia and esophagitis. The increase of p53 expression was greater in dysplasia associated with carcinoma than in non-carcinoma-associated dysplasia, suggesting that the expression of p53 shown by immunohistochemistry is an important biomarker in esophageal carcinogenesis in pre-cancerous lesions (12).

Table II. Kappa test to describe the intensity of agreement among the expression of three proteins simultaneously and association between protein expression, two by two, by Fisher's exact test on esophageal mucosa, in each evaluated group.

Group	p53		p16		Fhit		Kappa	Fisher exact test (P-value)		
	Altered	Normal	Altered	Normal	Altered	Normal		p53 x p16	p53 x Fhit	p16 x Fhit
NM	0 (0%)	10 (100%)	6 (60%)	4 (40%)	1 (10%)	9 (90%)	-0.222 ^{ns}	0.018*	1.0 ^{ns}	0.0573 ^{ns}
CD	1 (7.7%)	12 (92.4%)	10 (76.9%)	3 (23.1%)	1 (7.7%)	12 (93.3%)	-0.272 ^{ns}	0.0010*	1.52 ^{ns}	0.001*
CM	6 (26.1%)	17 (73.9%)	17 (73.9%)	6 (26.1%)	2 (8.7%)	21 (91.3%)	-0.268 ^{ns}	0.0028*	0.2427 ^{ns}	<0.0001*
CE	12 (52.2%)	11 (47.8%)	22 (95.7%)	1 (4.3%)	8 (34.8%)	15 (65.2%)	-0.238 ^{ns}	0.0017*	0.3726 ^{ns}	<0.0001*
ESCC	11 (100%)	0 (0%)	10 (90.9%)	1 (9.1%)	2 (18.2%)	9 (81.8%)	-0.310 ^{ns}	1.0 ^{ns}	0.0002*	0.0019*

NM, normal mucosa; CD, Chagas disease patients without megaesophagus; CM, chagasic megaesophagus; CE, chronic esophagitis; ESCC, esophageal squamous cell carcinoma. Immunostain: Altered, p53+ ≥ 3%, p16 and Fhit (absent/weak); Normal, p53+ ≤ 3%, p16 and Fhit (moderate/strong). Kappa test: ^{ns} Kappa value <0, no agreement between protein expressions (p53 vs. p16 vs. Fhit). Fisher exact test: ^{ns}no significant association; **p*<0.05, statistical association between protein expression.

In an Iranian study of familial and sporadic esophageal carcinoma, it was noted that 64.3% of family members studied had the *CDKN2A/P16* gene hypermethylated in the promoter region. In sporadic esophageal carcinoma, *P16* hypermethylation was observed in 73.3% of tumor cases; in samples of serum and blood, of the same patients, hypermethylation was found in 26.6% and 43.3%, respectively. These results indicated that *P16* could be used as a biomarker in the early identification of esophageal cancer in high-risk populations and in cases of family history (31). Therefore, both the occurrence of mutation and methylation of DNA, as the allelic loss (LOH) at *P16*, appear to play an important role in esophageal carcinoma development (31, 32). However, in the present work, analysis of the expression of p16 in the patients with benign esophageal lesions and carcinoma was not consistent, since no changes were found between these groups.

The abnormal expression of Fhit has been described as a common event in the early stage of esophageal carcinoma development, with complete loss or reduction of the protein expression, increasing along with histopathological severity, such as dysplasia to carcinoma *in situ* and invasive carcinoma (18, 19). However, no difference was found for Fhit expression in the esophageal lesions in the present study.

Smokers and alcoholic patients exhibit altered p53 expression, as shown by immunohistochemistry (7, 11). Excessive consumption of alcohol and tobacco are related to higher rates of loss of Fhit expression than are those of low consumption, confirming the finding that the *FHIT/FRA3B*

locus is susceptible to damage caused by environmental carcinogens (18, 20). However, analysis of all the groups the using the Fisher's exact test showed no association between the changes in the expression of the analyzed proteins and the demographic and clinicopathological parameters (sex, age, smoking and alcohol consumption).

According to the literature, the regulator proteins of the cell cycle are controlled by tumor suppressor genes and oncogenes; this interaction works in the regulation of cell cycle progression (33). The overexpression of p53 protein and loss of expression of Fhit and p16 proteins were reported by Chang *et al.* (34) in 64%, 75% and 81%, respectively, of cases of invasive esophageal carcinoma, indicating that expression changes in these proteins are related to neoplasia development. However, in our study, Kappa test analysis showed no agreement in the expression levels of these proteins, even though Fisher's exact test indicated an association between p53 and p16 (CD, CM and CE), p53 and Fhit (ESCC), p16 and Fhit (CD, CM, CE and ESCC) protein expression. In cells, thousands of molecules act together, but we cannot expect a perfect correlation among specific ones, which may explain the lack of agreement among the three proteins when examined simultaneously. It should also be considered that benign lesions such as CE and megaesophagus should not present multiple genetic alterations similar to carcinomas which show an accumulation of different alterations, involving both oncogenes and tumor suppressor genes.

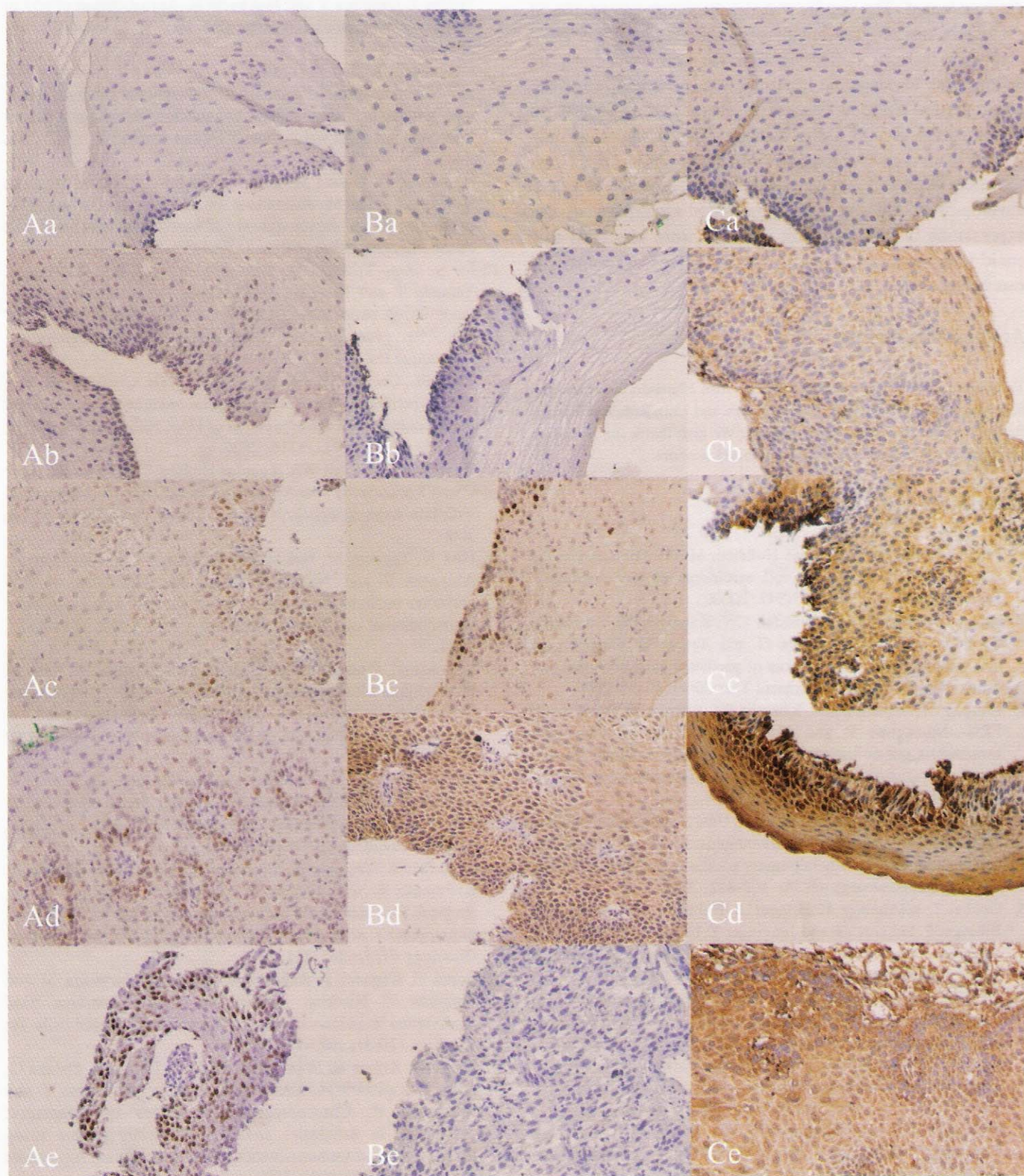


Figure 1. Immunohistochemical stain in esophageal mucosa. A, p53 protein: a, NM, negative; b, CD, negative; c, CM, p53+; d, CE, p53+ and e, ESCC, p53+. B, p16 protein: a, NM, weak; b, CD, absent; c, CM, cytoplasm moderate and nuclear strong; d, CE, moderate and e, ESCC, absent. C, Fhit protein; a, NM, weak; b, CD weak; c, CM, moderate; d, CE, strong and e, ESCC, strong. NM, normal mucosa; CD, Chagas disease patients without megaesophagus; CM, chagasic megaesophagus; CE, chronic esophagitis; ESCC, esophageal squamous cell carcinoma; Magnifications $\times 200$.

The present study of p53, p16 and Fhit protein expression in esophagitis and megaesophagus patients without cancer is unprecedented. The results support the involvement of p53 protein in the progression of esophageal carcinoma from precursor lesions, indicating that the cell cycle may be delayed due to persistent inflammation (esophagitis and the accumulation of food caused by megaesophagus) and so increases the risk of DNA damage. This association may gradually emerge as a sequence of severity of injuries (CM, CE and ESCC) and may be related to increased risk of tumor development. However, no significant alterations in the expressions of p16 and Fhit proteins were observed that could indicate a major role in the early steps of esophageal carcinogenesis.

Acknowledgements

The authors are grateful to Dr. Antonio José Manzato for statistical help and to Dr. Sebastião Roberto Taboga and Luiz Roberto Faleiros for help with the histological sections. We also thank the Brazilian agencies FAPESP and CNPq for financial support.

References

- Mandard AM, Hainaut P and Hollstein M: Genetic steps in the development of squamous cell carcinoma of the esophagus. *Mutation Res* 462(2-3): 335-342, 2000.
- Pisegna J, Holtmann G, Howden CW, Katelaris PH, Sharma P, Spechler S, Triadafilopoulos G and Tytgat G: Oesophageal complications and consequences of persistent gastro-oesophageal reflux disease. *Aliment Pharmacol Ther* 20(9): 47-56, 2004.
- Murphy SJ, Anderson LA, Johnston BT, Fitzpatrick DA, Watson PRG, Monaghan P and Murray LJ: Have patients with esophagitis got an increased risk of adenocarcinoma? Results from a population-based study. *World J Gastroenterol* 11(46): 7290-7295, 2005.
- Bektas A, Yasa MH, Kuzu I, Dogan I, Unal S and Ormeci N: Flow cytometric DNA analysis and immunohistochemical p53, PCNA and histopathologic study in primary achalasia: preliminary results. *Hepatogastroenterology* 48(38): 408-412, 2001.
- Gockel I, Kämmerer P, Brieger J, Heinrich UR, Mann WJ, Bittinger F, Eckardt VF and Junginger T: Image cytometric DNA analysis of mucosal biopsies in patients with primary achalasia. *World J Gastroenterol* 12(19): 3020-3025, 2006.
- McCabe ML and Dlamini Z: The molecular mechanisms of oesophageal cancer. *Int Immunopharmacol* 5: 1113-1130, 2005.
- Putz A, Hartmann AA, Fontes PR, Alexandre CO, Silveira DA, Klug SJ and Rabs HM: TP53 mutation pattern of esophageal squamous cell carcinomas in a high-risk area (Southern Brazil): role of life-style factors. *Int J Cancer* 98(1): 99-105, 2002.
- Pekarsky Y, Zanesi N, Palamarchuk A, Huebner K and Croce CM: *FHIT*: From gene discovery to cancer treatment and prevention. *Lancet Oncol* 3: 748-754, 2002.
- Kato H, Yoshikawa M, Fukai Y, Tajima K, Masuda N, Tsukada K, Kuwano H and Nakajima T: An immunohistochemical study of p16, pRb, p21 and p53 proteins in human esophageal cancers. *Anticancer Res* 20(1A): 345-349, 2000.
- Chino O, Kijima H, Shimada H, Nishi T, Tanaka H, Kise Y, Kenmochi T, Himeno S, Machimura T, Tanaka M, Inokuchi S, Tajima T, Osamura Y and Makuuchi H: Accumulation of p53 in esophageal squamous cell carcinoma. *Int J Mol Med* 8(4): 359-363, 2001.
- Fagundes RB, Melo CR, Putten AC, Moreira LF, de Barros SG: p53 Immunoexpression: An aid to conventional methods in the screening of precursor lesions of squamous esophageal cancer in patients at high risk? *Cancer Detect Prev* 29(3): 227-232, 2005.
- Kawakubo H, Ozawa S, Ando N, Kitagawa Y, Mukai M, Ueda M and Kitajima M: Alterations of p53, cyclin D1 and pRB expression in the carcinogenesis of esophageal squamous cell carcinoma. *Oncol Rep* 14(6): 1453-1459, 2005.
- Ito S, Ohga T, Saeki H, Watanabe M, Kakeji Y, Morita M, Yamada T and Maehara Y: Promoter hypermethylation and quantitative expression analysis of CDKN2A (*p14^{ARF}* and *p16^{INK4a}*) gene in esophageal squamous cell carcinoma. *Anticancer Res* 27(5A): 3345-3353, 2007.
- Chen PL, Cheng YW, Chiang CC, Tseng SH, Chau PS and Tsai YY: Hypermethylation of the *p16* gene promoter in pterygia and its association with the expression of DNA methyltransferase 3b. *Mol Vis* 17(12): 1411-1416, 2006.
- Kempster S, Phillips WA, Baidur-Hudson S, Thomas RJ, Dow C and Rockman SP: Methylation of exon 2 of *p16* is associated with late-stage oesophageal cancer. *Cancer Lett* 150(1): 57-62, 2000.
- Hibi K, Taguchi M, Nakayama H, Takase T, Kasai Y, Ito K, Akiyama S and Nakao A: Molecular detection of *P16* promoter methylation in the serum of patients with esophageal squamous cell carcinoma. *Clin Cancer Res* 7(10): 3135-3138, 2001.
- Takeuchi H, Ozawa S, Ando N, Kitagawa Y, Mukai M, Ueda M and Kitajima M: Further evidence that altered *p16/CDKN2* gene expression is associated with lymph node metastasis in squamous cell carcinoma of the esophagus. *Oncol Rep* 8(3): 627-632, 2001.
- Mori M, Mimori K, Shiraishi T, Alder H, Inoue H, Tanaka Y, Sugimachi K, Huebner K and Croce CM: Altered expression of Fhit in carcinoma and precarcinomatous lesions of the esophagus. *Cancer Res* 60(5): 1177-1182, 2000.
- Kitamura A, Yashima K, Okamoto E, Andachi H, Hosoda A, Kishimoto Y, Shiota G, Ito H, Kaibara N and Kawasaki H: Reduced Fhit expression occurs in the early stage of esophageal tumorigenesis: no correlation with p53 expression and apoptosis. *Oncology* 61(3): 205-211, 2001.
- Soma T, Kaganai J, Kawabe A, Kondo K, Imamura M and Shimada Y: Nicotine induces the fragile histidine triad methylation in human esophageal squamous epithelial cells. *Int J Cancer* 119(5): 1023-1027, 2006.
- Chino O, Kijima H, Shimada H, Nishi T, Tanaka H, Oshiba G, Kise Y, Kajiwarra H, Tsuchida T, Tanaka M, Tajima T and Makuuchi H: Clinicopathological studies of esophageal carcinoma in achalasia: analysis of carcinogenesis using histological and immunohistochemical procedures. *Anticancer Res* 20(5C): 3717-3722, 2000.
- Manoel-Caetano FS, Borim AA, Caetano A, Cury PM and Silva AE: Cytogenetic alterations in chagasic achalasia compared to esophageal carcinoma. *Cancer Genet Cytogenet* 149(1): 17-22, 2004.

- 23 Blanchard C, Wang N, Stringer KF, Mishra A, Fulkerson PC, Abonia JP, Jameson SC, Kirby C, Konikoff MR, Collins MH, Cohen MB, Akers R, Hogan SP, Assa AH, Putnam PE, Aronow BJ and Rothenberg ME: Eotaxin-3 and uniquely conserved gene-expression profile in eosinophilic esophagitis. *J Clin Invest* 116(2): 536-547, 2006.
- 24 Huiping C, Jonasson JG, Agnarsson BA, Sigbjornsdottir BI, Huebner K and Ingvarsson S: Analysis of the fragile histidine triad (*FHIT*) gene in lobular breast cancer. *Eur J Cancer* 36: 1552-1557, 2000.
- 25 Bodner-Adler B, Bodner K, Czerwenka K, Kimberger O, Leodolter S and Mayerhofer K: Expression of p16 protein in patients with uterine smooth muscle tumors: an immunohistochemical analysis. *Gynecologic Oncol* 96: 62-66, 2005.
- 26 GRAPHPAD, [Internet]. Accessed on: April 3rd, 2007. <http://www.graphpad.com/instat>
- 27 LEE, Laboratório de Epidemiologia e Estatística. Análise de Concordância kappa [Internet]. Accessed on: April 3rd, 2007. <http://www.lee.dante.br/pesquisa/kappa/index/html>
- 28 Brasileiro Filho G: Patologia das principais doenças tropicais no Brasil. In: Bogliolo Patologia (Brasileiro-Filho G). Rio de Janeiro, Guanabara Koogan, 7^a ed., pp. 1492, 2006.
- 29 Fuji T, Yamana H, Sueyoshi S, Fujita H, Tanaka Y, Kubota M, Toh U, Mine T, Sasahara H, Shirouzu K, Kato S and Morimatsu M: Histopathological analysis of non-malignant and malignant epithelium in achalasia of the esophagus. *Dis Esophagus* 13(2): 110-116, 2000.
- 30 Pajceki D, Zilberstein B, dos Santos MAA, Ubriaco JA, Quintanilha AG, Ceconello I and Gama-Rodrigues J: Megaesophagus microbiota: A qualitative and quantitative analysis. *J Gastroint Surg* 6(5): 723-729, 2002.
- 31 Abbaszadegan MR, Raziee HR, Ghafarzadegan K, Shakeri MT, Afsharnejad S and Ghavamnasiry MR: Aberrant p16 methylation, a possible epigenetic risk factor in familial esophageal squamous cell carcinoma. *Int J Gastrointest Cancer* 36(1): 47-54, 2005.
- 32 Krüger S, Mahnken A, Kausch I and Feller AC: P16 Immunoreactivity is an independent predictor of tumor progression in minimally invasive urothelial bladder carcinoma. *Eur Urology* 47(4): 463-467, 2005.
- 33 Lam AK: Molecular biology of esophageal squamous cell carcinoma. *Crit Rev Oncol Hematol* 33(2): 71-90, 2000.
- 34 Chang MS, Lee HS, Lee BL, Kim YT, Lee JS and Kim WH: Differential protein expression between esophageal squamous cell carcinoma and dysplasia, and prognostic significance of protein markers. *Pathol Res Pract* 201(6): 417-425, 2005.

Received July 14, 2008

Revised October 7, 2008

Accepted October 1, 2008

Capítulo III

CAPÍTULO III: Expression of Ki67 antigen and caspase-3 protein in benign lesions and esophageal carcinoma and relationship with p53 protein.

Authors: Marilanda Ferreira Bellini¹; Patrícia Maluf Cury², Ana Elizabete Silva¹

Authors Addresses:

¹ UNESP, São Paulo State University, Department of Biology, Campus São José do Rio Preto, SP, Brazil

² FAMERP – Medicine School, Department of Pathology, São José do Rio Preto, SP, Brazil

Correspondences to: Ana Elizabete Silva, IBILCE/UNESP – Departamento de Biologia, Rua Cristóvão Colombo, 2265, São José do Rio Preto, SP, Brazil - CEP. 15054-000; phone 55 - 17 - 32212384
e-mail: anabete@ibilce.unesp.br

Artigo original a ser submetido e redigido nas normas da revista

APOPTOSIS (e-ISSN: 1573-675X)

Resumo

O desbalanço entre proliferação e morte celular é um dos fatores chave da tumorigênese. O presente estudo teve por objetivos verificar alterações nos níveis de proliferação celular e apoptose em lesões benignas esofágicas em comparação com carcinoma esofágico e estabelecer associações com a expressão da proteína p53, estimada em estudo prévio. Ensaio de immuno-histoquímica foram realizados para o antígeno Ki67 e proteína caspase-3 (CPP32) em mucosa esofágica de pacientes com Doença de Chagas sem (CD) e com megaesôfago (CM), pacientes com esofagite crônica (CE), pacientes com carcinoma esofágico (ESCC) e em mucosa normal (NM). Associações com a expressão de p53, estimada em estudo anterior, também foram realizadas. O índice de marcação de Ki67 (LI) foi similar em todos os grupos (variação de 30,1% a 48%), não sendo observadas diferenças significantes. A frequência de positividade de CPP32 foi similar nos grupos CD (30,8%), CM (30,4%) e CE (34,8%), mas aumentada em ESCC (55,5%), embora sem diferenças significantes. Nenhuma associação entre os níveis de CPP32, Ki67 e expressão da proteína p53 foram observadas, nos diferentes grupos. Desta forma, não há evidências de alterações nos níveis de proliferação celular e apoptose nas lesões benignas estudadas.

Palavras-chave: CPP32, Ki67, megaesôfago chagásico, esofagite crônica, carcinoma esofágico.

Abstract

One key factor in the tumorigenesis is the imbalance between cell proliferation and death. The present study aimed to evaluate alterations of apoptosis and cell proliferation in esophageal benign lesion comparing to esophageal carcinoma and establish relationship with p53 expression estimated in previous study. Immunohistochemistry was performed for expression of caspase-3 protein (CPP32) and Ki67 antigen in the esophageal mucosa from patients with Chagas disease without (CD) and with megaesophagus (CM), patients with chronic esophagitis (CE), esophageal carcinoma (ESCC) and in normal mucosa (NM). The Labeling Index (LI) for Ki67 was similar in every group (range: 30.1% - 48%) and was not statistically significant. The positive CPP32 immunostaining was observed in similar frequencies in the groups of CD (30.8%), CM (30.4%) e CE (34.8%), but increased in ESCC (55.5%), though no significant differences. No associations among the levels of CPP32, Ki67 and p53 expression were observed in the different groups. Thus are not evident changes in in benign studied lesions.

Key Words: CPP32, Ki67, chagasic megaesophagus, chronic esophagitis, esophageal squamous cell carcinoma

Introduction

The cancer development involves many genes that control growth, cell proliferation and homeostasis of the tissue by apoptosis [1]. After an inflammatory process or the toxic effects of carcinogens the cell proliferation increases the probability of new genetically altered cells [2]. There is a tendency of neoplastic population increase proliferative capacity and escape the control mechanisms of normal growth. With the increased cell proliferation, the mutant progeny arises as a result of genomic instability, where the majority does not survive due to immune selection, or apoptosis metabolic changes [3].

Many of the biochemical and morphological alterations that occur during apoptosis is consequence from cysteine proteases family called caspase, which in turn cleave the various substrate proteins that account for apoptosis. Caspase-3, also known as CPP32, YAMA, or apopain, is probably the one that so far best correlates with apoptosis. Caspase-3-mediated proteolysis, which is initiated by the multiple different stimuli (endogenous and exogenous) that induce apoptosis, is a critical element of the apoptotic process [4]. Analysis of the expression of caspase-3 from primary esophageal squamous cell carcinoma (ESCC) showed that 60% of the cases were positive [5], and that caspase-3 was associated with a favorable prognosis. Caspase-3 was suggested as a new prognostic factor in ESCC indicating that biomarkers related apoptosis can be used as predictive in advance and prognosis of ESCC [6, 7, 8].

The assessment of cell proliferation by the detection of Ki67 antigen in neoplastic cell populations has been shown to be of prognostic value. There is a strong correlation between low or high Ki67 index and low or high-grade histopathology of neoplasms [8]. The progressive increase of Ki67 antigen from dysplasia to ESCC, suggested that this antigen is an effective biomarker in the squamous epithelium of esophagus [9, 10, 11].

TP53 tumor suppressor gene, which acts on apoptosis and cell proliferation, is the major mutated gene in human neoplasm. Mutations in this gene may fail both to arrest of cell cycle as to cause cell death in cancer cells, because the mutated form of p53 is ineffective in triggering apoptosis [12].

Esophageal carcinogenesis follows the multistep model involving esophagitis, atrophy, dysplasia, carcinoma *in situ*, and finally invasive carcinoma [13, 14]. Thus is important to evaluated benign lesions with precancerous potential as esophagitis and megaesophagus (esophagus dilation), the later as a consequence of Chagas disease, which there are few genetic studies that investigated changes of their cell cycle, as apoptosis and cell proliferation. The aim of this study was to evaluate the levels of cell proliferation (Ki67) and apoptosis (CPP32) and their associations with expression of p53 protein, estimated in previous study [15] in benign and possible precursor lesions, as chagasic megaesophagus and chronic esophagitis in cancer-free patients and ESCC patients.

Samples and Methods

Samples

A total of 78 specimens of paraffin embedded esophageal tissue were obtained from patients who underwent middle and distal esophageal biopsies, before any chemo- or radiotherapy treatment, at Pathology Section, Hospital de Base (São José do Rio Preto, SP, Brazil) at the period between 2003 and 2006. Twenty-three specimens were proceeding from Chagas Disease patients with chagasic megaesophagus (CM), 13 with Chagas Disease (CD) but without megaesophagus, 23 from chronic esophagitis (CE) patients, and 9 from esophageal squamous cell carcinoma (ESCC). Both patients with Chagas Disease and chronic

esophagitis were esophageal cancer-free. Esophageal mucosa from 10 healthy patients were obtained and diagnosed as histologically normal (NM).

In the NM, with histologically normal esophageal mucosa, the majority of subjects were female, aged between 26 and 67 years ($\bar{x} \pm SE = 42.4 \pm 4.81$), of which 40% of them have alcoholic habit and 30% have smoking habit. Of the 36 chronic chagasic patients, 13 did not have megaesophagus (CD), the majority being female, aged between 45 and 79 years ($\bar{x} \pm SE = 61.3 \pm 2.85$). Among chagasic without megaesophagus, 30.8% were alcoholic and 38.5% were smokers. In this group, 53.8% developed other complications related to Chagas disease - (5 / 13) and megacolon (2 / 13) chagasic cardiomyopathy, and most (76.9% - 10/13) had mild to moderate esophagitis. The other 23 patients had chronic chagasic megaesophagus (CM) and most were male, aged 57 to 83 years ($\bar{x} \pm SE = 64.3 \pm 2.08$). As the classification of megaesophagus, 4.3% (1 / 23) of cases were grade I, 13% (3 / 23) grade II, 43.5% (10/23) grade III and 39.2% (9 / 23) Grade IV. Among patients with chagasic megaesophagus, 34.8% (8 / 23) had alcoholic habit and 56.5% (13/23) smoking. Among other complications of Chagas' disease, four patients (17.4%) developed megacolon, and 34.8% have heart disease. Except for six cases, all other patients had esophagitis: 23.5% mild, 47.1% moderate and 29.4% severe. In the group CE group, about half were female or male, aged 40 to 70 years ($\bar{x} \pm SE = 50.5 \pm 1.99$). Only 4.3% of the cases were drinkers and 17.4% were smokers. In contrast, the group with squamous cell carcinoma of the esophagus was formed by 8 males and one female, with ages ranging from 42 to 69 years ($\bar{x} \pm SE = 56.5 \pm 2.68$). Of these, most are drinker and smoker (72.7% and 81.8%, respectively). In this group, three patients are chagasic, of which two had chagasic megaesophagus associated with carcinoma.

The study was approved by Brazilian National Research Ethics Committee (CONEP).

Immunohistochemical Assay

Consecutive 4µm-thick sections were cut from each trimmed paraffin block, and mounted in glass slides pre-treated with 3-aminopropyl-triethoxysilane/acetone solution. In brief, following deparaffinization, sections were re-hydrated, treated with citrate buffer, at 96°C, for 30 min.; and treated with 3% H₂O₂ in methanol (v/v) for 30 min. to block the endogenous peroxidases. The sections were then incubated for 1 hour, at room temperature, with specific antibodies: CPP32 (clone JHM-62, Novocastra, Newcastle, UK, 1:100) and Ki67 antigen (clone MM1, Novocastra, Newcastle, UK, 1:200). The slides were incubated with secondary antibody; next, slides were incubated polymer to CPP32 (Novolink Polymer, Novocastra, Newcastle, UK,) or streptavidin-biotin peroxidase to Ki67 (Dako Cytomation Kit, Strept ABCComplex/HRP, Dako, Glostrup, Denmark), following the manufacturer's instructions. The immunostaining was visualized with 3,3' diaminobenzidine tetrahydrochloride (DAB) containing 0,005% H₂O₂, and counterstained with hematoxylin. Negative controls were established by replacing the primary antibody with buffer solution. Tonsil tissues were used as positive controls for both antibodies.

The polyclonal antibody (NCL-Ki67p) labels Ki67 antigen in the granular components of the nucleolus during late G1, S, G2 and M phases [9]. The positive immunostain for Ki67 antigen was brown nuclear staining and the negative was the absence of nuclear staining. Five hundred cells were counted for each sample. Ki67 was considered positive when >10% of cells showed nuclear reactivity [16, 17]. The Labeling Index (LI) for Ki67 antigen (the ratio of cells positively stained for Ki67 on the total of cells counted for each case [12] was calculated.

For CPP32, all tissue extension was examined for each sample. Immunostaining for CPP32 (brown cytoplasm staining) was graded by intensity of staining as negative: (-) absent brown staining and (+) weakly stained; or as positive: (++) moderately stained and (+++) strongly stained [4].

All analysis was done under a light microscope (x400 magnification). Areas that were poorly preserved, crushed, cauterized, folded, or retracted were specifically avoided.

Statistical Analysis

Descriptive statistics, Kolmogorov and Smirnov method for normality distribution, Kruskal-Wallis Test for CPP32 and Ki67; and Analysis of Variance (ANOVA) with Tukey-Kramer Multiple Comparisons post-test for Ki67 antigen were used to determine statistical significance. Fisher's Exact Test was performed to establish associations between apoptosis, cell proliferation and p53 expression, two by two. The level of significance was set as $P < 0.05$. The statistical analysis was carried out with GraphPad Instat 3 computer software [18].

Results

The LI for Ki67 antigen for each group was as follows: 30.1% of NM, 30.9% of CD, 42.3% of CM; 44.6% of CE and 48.4% of ESCC in 500 cells analyzed per case (Table 1). The Kolmogorov and Smirnov method indicated normality distribution and then ANOVA with Tukey-Kramer Multiple Comparison post-test was performed among the groups, but no significant difference was observed.

One hundred percent of the cases in the NM, CD, CE and ESCC groups were classified as Ki67 positive, since they showed more than 10% of cells positively immunostained for the antigen, while in the CM

group 91.3% of cases were Ki67 positive. The statistical analyses for the positive cases, by Kruskal-Wallis Test, did not indicate any significant difference among the groups (Table 1).

Positive cytoplasmatic immunostaining for CPP32 was observed in a few cases in the normal mucosa group 20% (2/10), which was interpreted as normal level of apoptosis. The positive CPP32 immunostaining was observed in similar frequencies in the groups of CD, CM and CE respectively, 30.8% (4/13), 30.4% (7/23), and 34.8% (8/23), but increased in ESCC group, 55.5% (5/9) (Table 1). Statistically significant differences were not observed using the test Kruskal-Wallis test among the groups.

The establishment of associations between CPP32, Ki67 and p53 expression (based on previous data, [15]) were performed by Fisher's Exact Test, indicating that there is no association between the cell regulation processes studied in the groups analyzed (Table 2), neither between the altered expression of p53 and CPP32 protein or Ki67 antigen in each studied group (Table 3). Moreover, significant associations were not observed between altered expression pattern of Ki67 antigen and CPP32 protein, with parameters such as age, gender, smoking and alcoholism (data not shown) at different groups.

Figure 1 illustrates the immunostaining of Ki67 antigen (nuclear) and CPP32 protein (cytoplasmic) in the esophageal mucosa of benign lesions and carcinoma.

Discussion

Recent studies have demonstrated that gene abnormalities of cell-cycle regulators that function in the transition from the G1 to S phase are associated with life style factors in ESCC [19], however, associations between these parameters, such as age, gender, smoking

and alcoholism, and the status of Ki67 antigen and CPP32 protein were not observed in the studied groups (data not shown).

In the present study, the Ki67 labeling index (LI) were higher in CM, CE and ESCC groups, than in the others, although both LI and positivity for Ki67 was not statistically significant among the different groups. However, ESCC showed higher expression of CPP32 than the others groups, indicating high level of apoptosis in tumor tissue. When the analysis of associations between the molecules (Ki67, CPP32 and p53) could be performed, no associations were found.

Tumor cell cycle analysis has indicated that tumors with a higher proliferation rate ($\geq 40\%$) show more aggressive clinical behaviors compared to tumors with a low proliferation rate ($< 40\%$) [20]. The Ki67 LI has been identified as a parameter of tumor proliferation. ESCC patients with a high Ki67 LI have lower postoperative survival rates; thus, a high Ki67 LI is one of the prognostic factors of ESCC [19, 21]. In our study, the Ki67 LI were greater than 40% in CM (14 cases, 60.8%), CE (11 cases, 47.8%) and ESCC (5 cases, 55.5%) groups, but did not show significant difference with the NM and CD groups (with Ki67 LI $\sim 30\%$). Despite some studies which indicate that the Ki67 LI is a powerful prognostic marker for patients with esophageal carcinoma [22, 23, 24], the results of the current study did not displayed increased Ki67 LI in the ESCC group compared to others groups analyzed.

High expression of tumor proliferation-related factors (Ki67, PCNA, and AgNOR), abnormalities of adhesion molecule (E-Cadherin, alpha-Catenin), activation of autocrine mechanism of growth factor (EGFR, TGF α , EGF), and DNA ploidy pattern, which is thought to be the result of an accumulation of genomic abnormalities are also prognostic factors for esophageal cancer [25]. Ki67-% was significantly higher in patients with erosive esophagitis and in patients with functional heartburn than in controls, concluding Ki67 evaluation provides quantitative and objective data on squamous epithelium proliferative activity [9, 11]. Meanwhile, the

present study did not find any statistical significant difference among the benign lesion and ESCC, neither among the normal mucosa. Though, the expression of Ki67 did not show significant differences in biomarker expression between carcinoma of the esophagus and its precursor lesions (mild, moderate and severe dysplasia) [20], what corroborate our results.

We observed an increased expression of CPP32 protein only in ESCC group (55.5%), but it was not significant. The positive staining in ESCC group should be arose from the high apoptotic activity in response to the severe injury, whereas in CD, CM and CE (positive in ~30% cases), it might be occur as a consequence of physiological and inflammatory process of esophageal epithelium. So the high expression of CPP32 protein in ESCC, in the current study, may indicate a favorable prognostic, according some studies that observed a relationship between moderate to strong CPP32 staining and better prognostic [6]. Moreover, caspase-3 revealed significant increase in the positivity pattern between dysplasia and their corresponding invasive cancer portion and may be involved in the progression from dysplasia to ESCC [26, 27]. In conclusion, the analysis of the apoptotic protein expression patterns would be valuable to develop rational strategies for early detection of lesions at risk in advance as well as for prevention and treatment of ESCC.

Wild type of p53 triggers apoptosis following DNA damage or induces p21WAF1/Cip1, which causes G1 arrest, allowing time for damaged DNA to be repaired. Therefore, examining the relationship among p53, cell proliferation and apoptosis may be critical to determine the progress of carcinogenesis [12, 28]. Beardsmore et al. (2003) [12] found relationship between p53 expression and apoptosis, or p53 expression and cell proliferation indexes when ESCC patients were treated with chemotherapy. But, in our study associations between the molecules CPP32, Ki67 and p53 could not be performed in all groups, because, in some cases, the combination of variables was zero. When the analysis was performed in the groups CD (CPP32 x p53); CM (Ki67 x p53;

Ki67 x CPP32 and CPP32 x p53) and CE (CPP32 x p53); we could not find any relationship among the expression of the molecules evaluated. These results also can be in consequence of reduced number of cases evaluated in each group.

The absence of association between Ki67 antigen and CPP32 protein could be related to an imbalance in cell defense mechanism, when the organism tries to interrupt the proliferation of damaged cell, decreasing cell proliferation and increasing the apoptosis levels, because close coordination of these two phenomena is essential not only for a regulation and normal physiology, but to disease prevention [4] and combat.

In conclusion, the role of the Ki67 antigen in ESSC seems not clear, because some authors believe in the increase of Ki67 expression following the severity of the lesion, while others do not. In the present study, we did not find any difference among the groups, indicating the need of further investigations. Our results did not show statistically difference of the CPP32 expression between the benign lesion groups and ESCC group. Also were not observed associations among the levels of CPP32, Ki67 and p53 expression. Thus it is not evident significant changes in apoptosis and cell proliferation in the studied esophageal benign lesions, at the tested conditions.

Acknowledgements:

The authors are grateful to Dr. Sebastião Roberto Taboga and Luiz Roberto Faleiros for help with histological sections and photo documentation. We also thank Brazilian agencies Fundação de Apoio à Pesquisa do Estado de São Paulo (FAPESP) and Conselho Nacional de Pesquisa e Desenvolvimento (CNPq) for financial support.

References:

- [1] Koch WM, Boyle JO, Mao L, et al (1994) TP53 gene mutations as markers of tumor spread in synchronous oral cancer. *Arch. Otolaryngol. Head Neck Surg.* 120:943-947.
- [2] Cheng KC, Loeb LA (1993) Genomic instability and tumor progression: mechanistic considerations. *Adv. Cancer Res.* 60:121-156.
- [3] Nowell PC (1976) Clonal evolution of tumor cell sub-populations. *Science* 194:23.
- [4] Krajewska M, Wang HG, Krajewski S et al (1997) Immunohistochemical analysis of in vivo patterns of expression of CPP32 (Caspase-3), a cell death protease. *Cancer Res.* 57(8):1605-1613.
- [5] Hsia Jy, Chen Cy, Chen Jt et al (2003). Prognostic significance of caspase-3 expression in primary resected esophageal squamous cell carcinoma. *EJSO* 29:44-48.
- [6] Sutter AP, Maaser K, Höpfner M et al (2002) Specific ligands of the peripheral benzodiazepine receptor induce apoptosis and cell cycle arrest in human esophageal cancer cells. *Int. J. Cancer.* 102:318–327.
- [7] Xue LY, Hu N, Song YM et al (2006) Tissue microarray analysis reveals a tight correlation between protein expression pattern and progression of esophageal squamous cell carcinoma. *BMC Cancer* 6: 296-311.
- [8] IHC World (2008) Ki67 Antibody Staining Protocol for Immunohistochemistry. In: Life Science Information Network. IHC World, LLC. Available via DIALOG
http://www.ihcworld.com/_protocols/antibody_protocols/ki67_novocastra.htm
 Accessed November 25th, 2008.
- [9] Mastracci L, Grillo F, Zentilin P et al (2006) Cell proliferation of squamous epithelium in gastro-oesophageal reflux disease: correlations with clinical, endoscopic and morphological data. *Aliment Pharmacol Ther.* 25:637-645.
- [10] Figueroa JD, Terry MB, Gammon MD et al. (2008) Cigarette smoking, body mass index, gastro-esophageal reflux disease, and non-steroidal anti-inflammatory drug use and risk of subtypes of esophageal and gastric cancers by P53 overexpression. *Cancer Causes Control.* in press.

- [11] Zhang GH, Su M, Tian DP et al (2008) Analysis of basement membrane structure and inflammation during the development of esophageal squamous cell carcinoma in the Chinese Chaoshan high risk region. *Cancer Invest.* 26(3):296-305.
- [12] Beardsmore DM, Verbeke CS, Davies CL et al (2003) Apoptotic and proliferative indexes in esophageal cancer: predictors of response to neoadjuvant therapy. *J Gastrointest Surg.* 7(1):77-86.
- [13] Muñoz N (1997) Le dépistage du cancer de l'oesophage est-il réalisable? *Semaines des Hôpitaux de Paris* 73:633-635.
- [14] Mandard AM, Hainaut P, Hollstein M (2000) Genetic steps in the development of squamous cell carcinoma of the esophagus. *Mutat Res.* 462:335-342.
- [15] Bellini MF, Leite KRM, Cury PM et al (2008) p53, p16 and Fhit proteins expressions in chronic esophagitis and chagas disease. *Anticancer Res.* 28(6A):3793-3800.
- [16] Miller RT (2003) Immunohistochemistry in Urothelial Dysplasia. In: *The Focus- Immunohistochemistry. ProPath.* Available via DIALOG http://www.ihcworld.com/_newsletter/2003/focus_jun_2003.pdf Accessed December 17th, 2008
- [17] Boone J, van Hillegersberg R, van Diest PJ et al (2008) Validation of tissue microarray technology in squamous cell carcinoma of the esophagus. *Virchows Arch.* 452:507–514.
- [18] Instat (2007) GraphPad Instat. Instant biostatistic. In: *GraphPad Software, Analyse, Graph and organize your data.* Available via DIALOG <http://www.graphpad.com/instat/instat.htm> Accessed April 03rd, 2007
- [19] Takeuchi H, Ozawa S, Ando N et al (2003) Cell-Cycle Regulators and the Ki-67 Labeling Index Can Predict the Response to chemoradiotherapy and the Survival of Patients With Locally Advanced Squamous Cell Carcinoma of the Esophagus. *Annals of Surgical Oncology*, 10(7):792–800.
- [20] Huang JX, Yan W, Song ZX et al (2005) Relationship between proliferative activity of cancer cells and clinicopathological factors in patients with esophageal squamous cell carcinoma. *World J Gastroenterol.* 19:2956-2959.

- [21] Horii N, Nishimura Y, Okuno Y et al (2001) Impact of neoadjuvant chemotherapy on Ki-67 and PCNA labeling indices for esophageal squamous cell carcinoma. *Int. J. Radiation Oncology Biol. Phys.* 49(2):527–532.
- [22] Youssef EM, Matsuda T, Takada N et al (1995) Prognostic significance of the MIB-1 proliferation index for patients with squamous cell carcinoma of the esophagus. *Cancer* 76:358–366.
- [23] Kawamura T, Goseki N, Koike M et al (1996) Acceleration of proliferative activity of esophageal squamous cell carcinoma with invasion beyond the mucosa: Immunohistochemical analysis of Ki-67 and p53 antigen in relation to histopathological findings. *Cancer* 77:843–849.
- [24] Ikeda G, Isaji S, Chandra B et al (1999) Prognostic Significance of Biologic Factors in Squamous Cell Carcinoma of the Esophagus. *Cancer*. 86 (8):1396–1405.
- [25] Shimada Y, Imamura M (1996) Prognostic factors for esophageal cancer--from the viewpoint of molecular biology. *Gan To Kagaku Ryoho* 23(8):972-981.
- [26] Kurabayashi A, Furihata M, Matsumoto M et al (2001) Expression of Bax and apoptosis-related proteins in human esophageal squamous cell carcinoma including dysplasia. *Mod Pathol.* 14(8):741-747.
- [27] Chang MS, Lee HS, Lee BL et al (2005) Differential protein expression between esophageal squamous cell carcinoma and dysplasia, and prognostic significance of protein markers. *Pathol Res Pract* 201(6): 417-425.
- [28] Okumura H, Natsugoe S, Nakashima S et al (2000) Apoptosis and cell proliferation in esophageal squamous cell carcinoma treated by chemotherapy. *Cancer Lett.* 158:211-216.

Table 1. Immunohistochemical and statistical analysis for Ki67 antigen and CPP32 protein in the different groups.

		Ki67		CPP32		
Groups	N	Labeling Index (LI)	Positive		Positive (++/+++)	
		$\bar{x} \pm SE$ (%) Range	n/N	%	n/N	%
NM	10	30.1 $\pm$ 2.63 ^{ns} 18.2 - 44	10/10	100.0 ^a	2/10	20.0 ^a
CD	13	30.9 $\pm$ 2.80 ^{ns} 12.0 – 42.0	13/13	100.0 ^a	4/13	30.8 ^a
CM	23	42.3 $\pm$ 3.82 ^{ns} 2.4 – 62.8	21/23	91.3 ^a	7/23	30.4 ^a
CE	23	44.6 $\pm$ 3.28 ^{ns} 20.6 – 76.4	23/23	100.0 ^a	8/23	34.8 ^a
ESCC	9	48.4 $\pm$ 7.12 ^{ns} 21.2 – 72.0	9/9	100.0 ^a	5/9	55.5 ^a
Statistical Analysis		ANOVA, p = 0.0120 Tukey-Kramer Multiple Comparison Test, p > 0.05	Kruskall-Wallis Test, p = 0.3035		Kruskall-Wallis Test, p = 0.5731	

NM, health patients; CD, Chagas Disease patients without megaesophagus; CM, chagasic megaesophagus; CE, chronic esophagitis; ESCC, esophageal squamous cell carcinoma; N, total of specimens; n, number of specimens positive immunostained; $\bar{x}$, average; SE, standard error. Statistical Analysis: groups following the same letter are not statistically different, on the same column; ^{ns}, there is not statistical difference.

Table 2. Association between expression of Ki67 antigen and CPP32 protein, by Fisher's Exact Test (p<0.05).

Groups	Ki67 +		Ki67 -		p value
	CPP32 +	CPP32 -	CPP32 +	CPP32 -	
NM	1 (10%)	9 (90%)	0 (0%)	0 (0%)	NP
CD	1 (7.69%)	12 (92.31 %)	0 (0%)	0 (0%)	
CM	6 (26.08%)	15 (65.22%)	0 (0%)	2 (8.70%)	1.000 ^{ns}
CE	12 (52.17%)	11 (47.83%)	0 (0%)	0 (0%)	NP
ESCC	5 (55.56%)	4 (44.44%)	0 (0%)	0 (0%)	NP

NM, health patients; CD, Chagas Disease patients without megaesophagus; CM, chagasic megaesophagus; CE, chronic esophagitis; ESCC, esophageal squamous cell carcinoma; NP, it was not performed because one row or column is filled with zeros and in this situations the analysis is impossible; ^{ns} it is not statistically significant

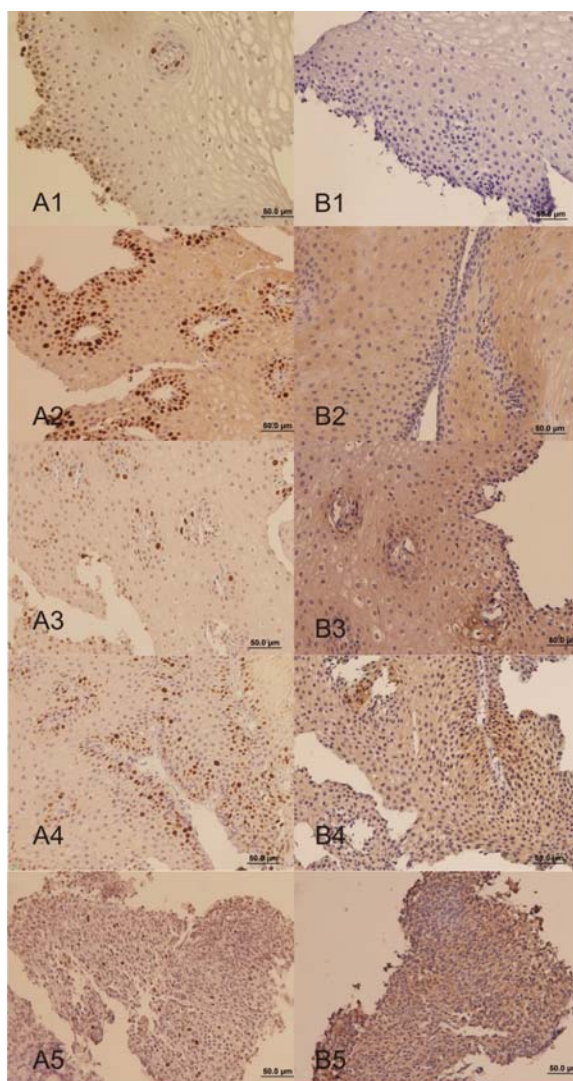
Table 3. Association between expression of Ki67 antigen and CPP32 protein with p53 protein ^[15], by Fisher's Exact Test (p<0.05).

Groups	Ki67 +		Ki67 -		Ki67xp53		CPP32 +		CPP32 -		CPP32xp53
	p53 +	p53 -	p53 +	p53 -	p53 +	p value	p53 +	p53 -	p53 +	p53 -	p value
NM	0 (0%)	10 (0%)	0 (0%)	0 (0%)	0 (0%)	NP	0 (0%)	1 (10%)	0 (0%)	9 (90%)	NP
CD	4 (30.8%)	9 (69.2%)	0 (0%)	0 (0%)	0 (0%)	NP	0 (0%)	4 (30.8%)	1 (7.7%)	8 (61.5%)	1.0000 ^{ns}
CM	6 (26.0%)	15 (65.2%)	0 (0%)	0 (0%)	0 (0%)	NP	2 (8.7%)	5 (21.7%)	4 (17.4%)	12 (52.2%)	1.0000 ^{ns}
CE	8 (34.8%)	15 (65.2%)	0 (0%)	0 (0%)	0 (0%)	NP	3 (13.0%)	5 (21.7%)	9 (39.2%)	6 (26.1%)	0.4003 ^{ns}
ESCC	9 (100%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	NP	5 (55.5%)	0 (0%)	4 (44.4%)	0 (0%)	NP

NM, health patients; CD, Chagas Disease patients without megaesophagus; CM, chagasic megaesophagus; CE, chronic esophagitis; ESCC, esophageal squamous cell carcinoma; NP, it was not performed because one row or column is filled with zeros and in this situations the analysis is impossible; ^{ns} it is not statistically significant.

Legend of figure:

Figure 1. Immunostaining of Ki67 antigen and CPP32 in esophageal mucosa. A, Ki67 antigen. B. CPP32 protein. 1., normal mucosa (Ki67+, CPP32 -), 2. chagasic without megaesophagus (Ki67+, CPP32 ++), 3. chagasic megaesophagus (Ki67+, CPP32 ++), 4. chronic esophagitis (Ki67+, CPP32 +), 5. esophageal squamous cell carcinoma (Ki67+, CPP32 +++). Ki67+, more than 10% of cell is positively immunostained. CPP32 -, absence of immunostaining; CPP32 +, weak immunostaining; CPP32 ++, moderate immunostaining; CPP32 +++, strong immunostaining. Magnification 200x



Capítulo IV

CAPÍTULO IV: Chromosomal imbalances are uncommon in Chagasic megaesophagus

Authors: Bellini, MF ^{1,2}; Manzato, AJ ³; Silva, AE¹; Varella-Garcia, M²

Authors Addresses:

¹ UNESP, São Paulo State University, Department of Biology, Campus São José do Rio Preto, SP, Brazil

² University of Colorado Denver, Department of Medicine/Medical Oncology, Aurora, Colorado, USA

³ UNESP, São Paulo State University, Department of Computer Sciences and Statistics, Campus São José do Rio Preto, SP, Brazil

Correspondence to: Ana Elizabete Silva,
IBILCE/UNESP – Departamento de Biologia, Rua Cristóvão Colombo,
2265, São José do Rio Preto, SP, Brazil - CEP. 15054-000;
phone 55 - 17 – 32212384.
e-mail:anabete@ibilce.unesp.br

Running Title: Chromosomal Imbalances in chagasic megaesophagus

Artigo original a ser submetido e escrito nas normas da revista

**CHROMOSOME RESEARCH : CYTOGENETICS, GENOMICS,
CHROMATIN AND THE NUCLEUS** (e-ISSN: 1573-6849)

Resumo

A doença de Chagas é uma doença parasitária tropical. Entre os pacientes na fase crônica, 6 a 7% desenvolvem mega síndromes do esôfago ou cólon. A dilatação do esôfago é conhecida como megaesôfago chagásico (CM) e uma de suas conseqüências tardias é o aumento do risco desses pacientes desenvolverem carcinoma esofágico (ESCC). Baseando-se na relação entre o CM e ESCC, investigamos, em cortes histológicos da mucosa esofágica de pacientes com CM e mucosa normal (NM), genes frequentemente não-balanceados descritos em ESCC, como *FHIT*, *TP63*, *PIK3CA*, *EGFR*, *FGFR1*, *MYC*, *CDKN2A*, *YES1*, *NCOA3*, e centrômeros 3, 7 e 9, como controles, por FISH (Hibridação *In Situ* Fluorescente). A análise citogenética nas camadas superficial e basal da mucosa esofágica não mostrou diferenças significantes para a maioria dos alvos investigados, exceto para *EGFR* no grupo CM, que apresentou freqüência menor de cópias na camada basal. O número total de cópias de *PIK3CA*, *CDKN2A* e CEP9 foi significativamente menor em CM que em NM. Alguns casos de CM mostraram níveis não-significantes de perdas para genes específicos: *TP63*, *FHIT*, *PIK3CA*, *EGFR*, *CDKN2A*, *YES1*; CEP9 ou de ganhos para *PIK3CA*, *TP63*, *FGFR1*, *MYC*, *CDKN2A* e *NCOA3*. Em conclusão, a observação de que alvos genômicos com desequilíbrios na carcinogênese esofágica não são significativamente afetadas em CM sugere que não será possível utilizar tais características como marcadores eficazes de risco para desenvolvimento de ESCC em CM.

Palavras-chave: megaesôfago chagásico, carcinoma de células escamosas de esôfago e FISH

Abstract

Chagas' disease is a human tropical parasitic disease. Among the chronic chagasic individuals, 6 to 7% develop mega syndromes of the esophagus or colon. The esophagus dilation is known as Chagas megaesophagus (CM) and one of the serious late consequences of CM is the increased risk for esophageal carcinoma (ESCC). Based on the association between CM and ESCC, we proposed to investigate in CM the genomic status of DNA targets frequently unbalanced in ESCC as *FHIT*, *TP63*, *PIK3CA*, *EGFR*, *FGFR1*, *MYC*, *CDKN2A*, *YES1* and *NCOA3*; and the centromere 3, centromere 7 and centromere 9 were used as control, by FISH. Analyses in the superficial and basal layers of the epithelial mucosa did not show differences among them, excepted for the gene *EGFR* in CM, which was deleted in the basal layer. The copy number of *PIK3CA*, *CDKN2A* and CEP9 were significantly lower in CM than in NM. Some CM cases had non-significant levels of deletions in *TP63*, *FHIT*, *PIK3CA*, *EGFR*, *CDKN2A*, *YES1*; CEP9 or gains at *PIK3CA*, *TP63*, *FGFR1*, *MYC*, *CDKN2A* and *NCOA3*. In summary, the findings that genomic imbalances common in esophageal carcinomas are not affected in CM suggest that these features will not be effective biomarkers for risk assessment of ESCC in CM

Key Words: chagasic megaesophagus, esophageal squamous cell carcinoma, FISH

Introduction

Chagas' disease is a human tropical parasitic disease which occurs in the Americas, particularly in South America (Lana; Tafuri, 2000). During the chronic phase, 6 to 7% of chagasic patients develop mega syndromes (muscular hypertrophy and dilation) of the esophagus or colon, probably because the protozoan *T. cruzi* destroys the mioenteric plexus (Crawford, 2004). In these digestive ways, muscular hypertrophy and dilation of the esophagus or colon are observed in advanced stages of the disease (Brasileiro-Filho, 2006).

The megaesophagus is consequence of achalasia characterized by the destruction or lack of intramural nerve plexus, which determines the absence of peristalsis and lack of openness of the lower esophageal sphincter in response to swallowing. In consequence, there is food retention, called esophageal stasis (Kraichely; Farrugia, 2006), which can determine the appearance of acanthosis, paraceratose and leukoplakia, possibly pre-cancerous lesions (Pinotti et al, 1996). One of the serious late consequences of chagasic megaesophagus is the increased risk (3% to 8%), of developing esophageal squamous cell carcinoma (ESCC) compared to when megaesophagus is not present (Bektas et al, 2001; Brüncher et al., 2001, Gockel et al, 2006). Also, ESCC develops in chagasic megaesophagus patients at a younger age than in those without this disease (Crawford, 2004). The detection of cancer in these patients is complicated because the symptoms are hidden by the severe dysphagia caused by megaesophagus (Chino et al, 2000), and the diagnosis is made frequently only in advanced stage (Haubrich; Schaffner; Berk, 1995) resulting in a poor prognosis (Brücher et al, 2001).

ESCC has been reported as the ninth most common malignancy and ranks the sixth most frequent cause of death over the world, but its incidence varies extremely among regions (Health A to Z, 2008; INCA, 2008) The American Cancer Society estimated around 16,470 new cases of esophageal carcinoma in the USA population for

2008 (American Cancer Society, 2008). The Brazilian National Institute of Research in Cancer (INCA) reported that esophageal cancer was the sixth in the cancer rank of mortality in 2000 with 5,307 deaths (INCA, 2008) and estimated about 10550 new cases in 2008 of this malignancy ranging among geographical areas from 1.04 to 19.07 in males and from 0.39 to 7.58 in 100,000 in females (INCA 2008).

Geographical variations and higher incidence in patients over 40-year old favors a multifactorial origin for ESCC, and the involvement of environmental factors such as smoking and alcoholic habits has been long known (Matsuo et al., 2001; Kuwano et al., 2005; Pelucchi et al., 2008). In addition to the environmental and pathogenic factors, genetic alterations, such as chromosomal aneuploidies, allelic deletions, activation of oncogenes and inactivation of tumor suppressor genes are associated with esophageal carcinogenesis (Kuwano et al., 2005; Daigo; Nakamura, 2008; D'Amico, 2008). Most of these phenomena are associated with genomic imbalances, such as gene amplification, over-representation or loss (Daigo; Nakamura, 2008; D'Amico, 2008).

In ESCC, different techniques of molecular cytogenetic have shown unbalanced genomic regions implicated with the amplification of some oncogenes and deletion of tumor suppressor genes (Kuwano et al., 2005; Daigo; Nakamura, 2008; D'Amico, 2008). In this study were selected *FHIT*, *TP63*, *PIK3CA*, *EGFR*, *FGFR1*, *MYC*, *CDKN2A*, *YES1* and *NCOA3* genes, frequently unbalanced in ESCC (Pack et al., 1999; Shinomyia et al., 1999; Du Plessis et al., 1999; Pimkhaokham et al., 2000; Yen et al., 2001; Ishizuka et al., 2002; Nakakuki et al., 2002; Arai et al., 2003; Shiomi et al., 2003; Yen et al., 2005; Su et al., 2006; Wang et al., 2006)

The amplification of the tumor suppressor gene *TP63* (3p28, whose protein plays in the development and maintenance of stratified epithelial tissues) has been described by Yen et al. (2003, 2005), in early stage of ESCC carcinogenesis but down-regulated in advanced

stage of disease. (Yen et al., 2005). However, losses at 3p and 9p, correspondent to the *FHIT* and *CDKN2A* respectively, were lost in some cases of ESCC (Du Plessis et al, 1999, Pack et al, 1999, Shinomiya et al, 1999, Pimkhaokham et al, 2000, Yen et al, 2001, Arai et al, 2003, Shiomi et al, 2003, Su et al, 2006, Wang et al, 2006).

Amplification of *PIK3CA* (3q26), whose protein represents the catalytic subunit, which uses ATP to phosphorylate phosphatidylinositol, was assessed ESCC (Yen et al. 2003; Yang et al., 2008, National Center for Biotechnology Information, 2009). FISH using BAC clones containing oncogene *PIK3CA* found that gene was amplified in 6 cell lines. Q-PCR analysis of primary tumors revealed amplification of *PIK3CA* in 100% of the cases (Yen et al., 2003). Positive correlation was found only between amplification and tumor size, lymph node metastasis and clinical stage. *PIK3CA* gene amplification was highly correlated with protein overexpression, suggesting that gene amplification is one important mechanism involved in *PIK3CA* overexpression (Yang et al., 2008).

Overexpression of membrane receptors has been reported in various cancers, and it has been suggested that it may be a poor prognostic factor in cases with solid tumors (Sugiura et al, 2007). *EGFR* (7p12) is a cell surface protein that binds to epidermal growth factor. Binding of the protein to a ligand induces receptor dimerization and tyrosine autophosphorylation and leads to cell proliferation (National Center for Biotechnology Information, 2009). Overexpression of *EGFR* is observed in many cancers, sometimes accompanied by gene amplification. By FISH, *EGFR* gene showed amplification in ESCC (Sunpaweravong *et al.*, 2005; Hanawa *et al.*, 2006). Also, *EGFR* gene overrepresentation (balanced gene and chromosome 7 polysomy) was common event in ESCC (Mimura *et al.*, 2005; Sunpaweravong *et al.*, 2005). The co-expression of both *aFGF* and *FGFR1* (8p12) was associated with a larger tumor area and poorer prognosis what suggests the membrane receptors

may promote proliferation of esophageal cancer cells in an angiogenesis-independent and autocrine manner, and may contribute to rapid growth of esophageal cancer on recurrence after esophageal resection (Sugiura et al, 2007).

Ishizuka et al. (2002) and Arai et al. (2003) detected amplification of *MYC* (8q24) by aCGH. *MYC* was found to be amplified in the 3 ESCC cell lines (Ishizuka et al., 2002) and in 9 of 20 tumors found on the microarray, but it was located on chromosomal regions that showed no increase in DNA copy number by CGH (Arai et al., 2003).

YES1 (18p11.31, protein has tyrosine kinase activity) amplification was detected by FISH, what suggests it is likely to be candidate target for 18p11.3 amplification and be associated with esophageal carcinogenesis (Nakakuki et al., 2002, National Center for Biotechnology Information, 2009).

NCOA3 (20q12), which the protein is a nuclear receptor coactivator that interacts with nuclear hormone receptors to enhance their transcriptional activator functions, has been observed overexpressed and with increased copy number in 46% and 13% of ESCCs, respectively (Xu et al., 2007). Overexpression of *NCOA3* was observed more frequently in primary ESCCs in late T stages than that in earlier stages, but no significant association of expression of *NCOA3* and status of lymph node metastases was observed. These results suggest that overexpression of *NCOA3* caused by genomic gain or other molecular mechanisms might provide a selective advantage for the development and local invasion of certain subsets of ESCC.

Despite the numerous genetic alterations reported in esophageal carcinogenesis, studies in benign lesions with precancerous potential as megaesophagus are scarce. This study investigated the genomic status in chagasic megaesophagus of genes frequently unbalanced in ESCC with the goal of identifying a potential panel of markers of risk to cancer. Among the as *FHIT*, *TP63*, *PIK3CA*, *EGFR*,

FGFR1, *MYC*, *CDKN2A*, *YES1* and *NCOA3* and three DNA targets: centromere 3, centromere 7 and centromere 9 used as control, by FISH assay.

Material and Methods

1. Subjects Characterization

Formalin-fixed, paraffin-embedded (FFPE) esophageal mucosa was obtained from 10 health individuals with histologically normal esophagus (NM) and 40 patients with diagnosis of chagasic megaesophagus (CM) who underwent middle and distal esophageal biopsies from 2000 to 2007 at the Hospital de Base of the São José do Rio Preto, SP, Brazil. The study was approved by the Institution Research Ethical Committee (CEP) and by the Brazilian National Research Ethics Committee (CONEP).

From 40 CM patients, 20 were male and 20 were female and the mean age was 62.5 years (range: 40 – 83 years). Twenty-nine were never-alcoholics, 3 were former-alcoholics (at least 5 years) and 8 were current alcoholics (at least 5 years); 18 were never-smokers, 6 were former-smokers (at least 5 years) and 16 were current smokers (at least 5 years). The histological grade of megaesophagus was classified as I to IV, based on the retention of contrast, diameter, tonicity of the lower sphincter and the length of the esophagus body (de Rezende; Lauer; de Oliveira, 1960). This study cohort included 5 patients with megaesophagus grade I, 7 with grade II, 17 with grade III and 11 with grade IV. The group group was followed-up until the end of this study and none individual developed esophageal squamous cell carcinoma. The CM group was followed-up until the end of this study and none individual developed esophageal squamous cell carcinoma.

The control group (NM) was composed by 10 health patients who were submitted to endoscopy under suspicion of dyspepsia, but the histopathological analysis has shown normal esophageal mucosa.

In this group, seven individuals were female and three were male, and the mean age was 42.4 (range: 26 - 67 years), where four were current alcoholics and 6 were never alcoholics; 3 were current smokers and 7 were never smokers. Despite the groups composed for 10 patients, only two of them was tested for each probe set.

2. Fluorescence In Situ Hybridization (FISH)

Serial 4µm-thick sections were cut from each trimmed paraffin block and mounted in glass slides pre-treated with 3-aminopropyl-triethoxysilane/acetone solution. Tissue sections were incubated at 56°C overnight, deparaffinized in Citrisolv (Fisher Brand, Cat #22-143975) washes (three times for 10 min), and dehydrated in 100% ethanol. After incubation in 2xSSC (Sodium chloride, sodium citrate solution, pH 7.0) at 75°C, sections were digested with proteinase K (0.25 mg/ml in 2xSSC, pH 7.0) at 45°C, rinsed in 2xSSC (pH 7.0) at room temperature for 5 min, and dehydrated in an ethanol series.

The following DNA probe sets were used: *EGFR/CEP7* (Abbott Molecular, Cat. # 32-191053), *P16/CEP9* (*CDKN2A*, Abbott Molecular, Cat. # 32-190078), *c-MYC* (Abbott Molecular, Cat. # 32-190006) and homebrew probes: *FGFR1* (RP11-350N15), *FHIT* (CTD-2196D15) / centromere 3 (p alpha 3.5), *TP63* (RP11 - 373I6) / *PIK3CA* (RP11 - 245C23) and *YES1* (RP11-769O8) / *NCOA3* (RP11-456N23). Each probe set was applied to the selected area on a designated slide. The hybridization areas were covered with glass coverslip and sealed with rubber cement. Co-denaturation of chromosomal and probe DNAs was performed at 85°C for 10 min and hybridization was allowed to occur in a humidified chamber at 37°C for 24 h for commercial probes and 48h for homebrew probes. After hybridization, the slides were washed twice in 2xSSC/0.3% NP-40 at 73°C for 2min, rinsed in 2xSSC at RT for 2min, dehydrated in 70%, 85% and 100% ethanol, allowed to air dry and

counterstained with 4', 6'-diamino-2-phenylindole - DAPI, (0.3 ug/ml in Vectashield Mounting Medium, Vector, Cat. # H-1200).

Microscopic analyses were performed in epifluorescence microscope. Fluorescence signals were scored using single band filters for DAPI, FITC (fluorescein), and Texas red, a double-band pass filter (FITC and Texas red) and a triple-band pass filter (DAPI, FITC, and Texas Red). Histological areas previously selected in the HE-stained sections were identified in the fluorescence in situ hybridization (FISH)-treated slides. Signals were scored in 100 epithelial nuclei per specimen, in at least four distinct areas: 50 nuclei in the superficial layer (larger cells, closer to the lumen) and 50 nuclei in the basal layer (darker HE-stained nuclei, smaller cells, closer to the muscular layer). The scoring was performed in both layers to check for differences between proliferate activity, since basal layer cells are in high proliferative activity, whereas the superficial layer cells are more mature.

For 3 genes, *FHIT*, *EGFR* and *CDKN2A* the experiments were performed including centromeric sequences of carrier chromosomes as an internal control, respectively chromosomes 3, 7 and 9. This design was necessary for *FHIT* and *CDKN2A* since these genes are known for their loss in cancer specimens.

3. Statistical Analysis

Descriptive statistics were calculated using an Microsoft Excel macro template previously validated including mean copy number per cell, standard deviation and frequency of cells with 1, 2 and ≥ 3 copies of each DNA target tested, as well as the ratio for gene/control probe when applicable. Comparisons between the superficial and basal layers were done by *t*-student paired test, and between the experimental groups were performed by *t*-student unpaired test; in both cases confidence level was established as 0.05 (Graphpad, 2008). Association between age, gender and life style factors (smoking and alcoholism), and megaesophagus

grade in the groups was compared by ANOVA and the comparisons for aneusomies were done by χ^2 test. One exploratory analysis using three-dimensional plots was performed for the percentages of genes status classes.

Results

The descriptive indexes (mean and standard deviation) for the genes *FHIT*, *PIK3CA*, *TP63*, *EGFR*, *FGFR1*, *MYC*, *CDKN2A*, *YES1*, *NCOA3* and centromeric sequences of chromosomes 3, 7 and 9, and the results of the statistical analyses for comparison of mean copy numbers between the superficial and basal layers in each group are presented in Table 1. The range of the mean in the CM group was 1.49 - 1.79 in the superficial layer and 1.45 - 1.79 in the basal layer. In the NM the mean frequency ranged 1.35 – 1.77 in the superficial layer and 1.61-1.78 in the basal layer. Superficial and basal layers did not show significant differences, excepted for *EGFR* in the CM group ($p=0.008$), that showed lower copy number in the basal layer. These results supported the combination of both layers for comparison of each gene between the CM and NM groups (Table 2). The total counting of copy number of the *PIK3CA*, *TP63*, *FGFR1*, *MYC*, *YES1* and *NCOA3* genes are not statistically different, when the groups CM and NM were compared. However, the copy number of the gene *CDKN2A* was significantly lower ($p<0,0001$) in the CM group compared to NM, the same was observed for the centromere 9 (CEP9) ($p=0.0002$).

The gene/centromere ratio, for *FHIT*/CEN3, *EGFR*/CEP7 and *CDKN2A*/CEP9, genes for which a control probe (centromeric sequence) was included in the assay, in all CM and NM specimens analyzed was balanced, ranging from 0.96 to 1.00, and statistically significant difference was not detected between the mean copy number for each gene and its centromere (Table 3).

In the CM group there was no significant differences on the mean copy number per cell for most genomic target according the histological grades of megaesophagus (I, II, III and IV) excepted for *MYC* and *YES1* (Table 4), Similarly, significant associations with parameters such as age and gender and life style factors (smoking and alcoholism), , at different groups, were also not observed (data not shown).

When frequencies of nuclei with balanced copy number or loss and gain copy number for each target were evaluated (Table 5), only *PIK3CA* showed higher frequency of cells with loss in CM compared to NM (36.4% vs. 28.5%). Statistical analysis was not performed for gain due to the scarcity of cells with this pattern.

An exploratory analysis using three-dimensional plots of the percentages of genes status classes (loss, balanced and gain) for individual patients is illustrated in Figures 1, 2 and 3. Several patients were identified harboring deletions in specifics targets: CM36 (*FHIT*), CM13 and CM19 (*PIK3CA*), CM 13 (*TP63*), CM30 (*EGFR*), CM12 (*CDKN2A*), CM7 (*YES1*), and CM16, CM34 and CM36 (*CEP9*). Other patients have shown genomic gain for specific targets: CM11 and CM28 (*PIK3CA*), CM6 and CM7 (*TP63*), CM3, CM6 and CM30 (*MYC*), CM30 (*FGFR1*), CM11 and CM16 (*CDKN2A*), CM36 (*NCOA3*), and CM11 and CM21 (*CEP9*). Some showing changes in more than one target. Interestingly, although no statistical difference was observed among the megaesophagus grades, excepted for *MYC* and *YES1*, most of the patients detected with loss or gain in the exploratory analyses were diagnosed with advanced megaesophagus grade III and IV.

The Figure 4 shows some FISH images for the unbalanced genes and centromeric targets in the megaesophagus specimens.

Monosomies involving centromeres 3, 7, and 9 were also investigated in each group (Table 6) and the cells were classified in three classes: <2 copies, 2 copies and >2 copies. The CM group showed significantly increased frequencies of cells with only one copy for

centromeres 7 and 9 (51% and 44%, respectively) in comparison to the NM group (28% and 23%, respectively). The CM group also showed numerically higher frequencies of cells with >2 copies of centromeres 7 and 9 (1.68% and 10.38%, respectively) than the NM group (0.50% and 0.00%, respectively), although it was not possible to perform statistical analysis.

Discussion

The esophageal squamous cell carcinoma is one of the most prevalent cancers worldwide and has multifactorial origin, in which the environment plays very significant role (Health A to Z, 2008), especially the abuse of alcohol and smoking (Matsuo *et al.*, 2001; Kuwano *et al.*, 2005; Pelucchi *et al.*, 2008). Besides the participation of environmental and pathologic factors, several genetic alterations are associated with ESCC, such as aneuploidies, allelic deletions, activation of oncogenes and inactivation of tumor suppressor genes (Kuwano *et al.*, 2005; Daigo; Nakamura, 2008; D'Amico, 2008).

In the present study, the analysis of genomic status in CM of DNA targets frequently unbalanced in ESCC as *FHIT*, *TP63*, *PIK3CA*, *EGFR*, *FGFR1*, *MYC*, *CDKN2A*, *YES1* and *NCOA3*; and the centromeres 3, 7 and 9 used as control, showed that the superficial and basal layers of esophageal mucosa are similar, except for lower *EGFR* copy number in the basal than in the superficial layer. The analysis for gene loss and of centromeres, indicated only *PIK3CA* with loss pattern and monosomies of the chromosomes 7 and 9 in the CM group. Despite the differences were not statistically significant, we detected CM cases with loss for *FHIT*, *CDKN2A*, *EGFR*, *TP63*, *PIK3CA*, *YES1* and centromere 9 and cases with gain for *PIK3CA*, *TP63*, *MYC*, *FGFR1*, *CDKN2A*, *NCOA3* and centromere 9 sequences, most of all associated to grades III and IV of megaesophagus. Although significant differences were detected on the

mean copy number only for *MYC* and *YES1* among the histological grades of megaesophagus.

Human esophageal carcinoma expresses multi-autocrine growth factors and hormones including *EGF*. Overexpression of epidermal growth factor receptor (*EGFR*) by tumor cells is closely correlated with tumor invasion and poor prognosis. The accumulation and interaction of several growth factors produced by tumor cells are necessary for the progression of human esophageal and gastric carcinomas (Yoshida et al, 1990).

Evidence suggests the existence of a new model of *EGFR* signaling pathway in which activated *EGFR* undergoes nuclear translocation and subsequently regulates gene expression and potentially mediates other cellular processes. Likely through its ability to upregulate gene expression, the nuclear *EGFR* pathway is associated with the major characteristics of more aggressive tumors such as increased proliferative potential, nitric oxide synthesis, and accelerated G1/S cell cycle progression (Lo; Hung, 2006). Increased expression of nuclear EGFR is linked to poor clinical outcome in patients with oropharyngeal squamous cell carcinomas (P syrri et al, 2005). In this study, decrease in the copy number of *EGFR* was found in the basal layer of CM in comparison to the superficial layer and in one CM case there was *EGFR* loss (not statistically significant), what could indicate that *EGFR* may be deleted in this benign lesion.

The loss of *CDKN2A* function, preventing the blocking of G1 phase appears to be a necessary event for the progression of pre-cancerous cells to malignancy (Mori *et al.*, 1994; Nuovo *et al.*, 1999; Li;Yang, 2003). In primary ESCC and cell lines, occurrence of point mutations or deletions in the *CDKN2A* gene have been observed in 16% of cases (Chen *et al.*, 2006), but the most frequent phenomenon responsible for *CDKN2A* inactivation is hypermethylation of the promoter region. In ESCC and pre-neoplastic lesions several studies have shown

hypermethylation of *CDKN2A* with frequencies ranging from 20% to 88%. This frequency increases gradually with the histological severity of the disease and is associated with poor prognosis (Fukuoka *et al.*, 2006; Guo *et al.*, 2006, Roth *et al.*, 2006; Guo *et al.*, 2007; Ito *et al.*, 2007; Fujiwara *et al.*, 2008; Wang *et al.*, 2008). The loss of p16 protein expression, detected by immunohistochemical techniques, has been observed in 50% to 97% of cases of ESCC (Kato *et al.*, 2000; Ralhan *et al.*, 2000; Takeuchi *et al.*, 2001; Chang *et al.*, 2005; Guo *et al.*, 2007; Fujiwara *et al.*, 2008). The current study could only detect the fraction of cases in which the loss of *CDKN2A* was due to physical deletion. This higher frequency of *CDKN2A* and centromere 9 losses in CM compared with NM, observed in the present study, is also a phenomenon that associates CM with the esophageal carcinogenesis process, because patients with idiopathic achalasia and chagasic megaesophagus, with or without esophageal carcinoma, showed changes in the expression of p16 (Chino *et al.*, 2000) and Bellini *et al.* (2008) observed a marginal decrease in the p16 expression in megaesophagus.

TP63 and *PIK3CA* were reported as oncogenic potential in a variety of carcinomas (Yen *et al.*, 2005). Gene amplification is one of the basic mechanisms that lead to overexpression of oncogenes and *PIK3CA* and *TP63* are described as amplified in ESCC (Pimkhaokoham *et al.*, 2002; Yen *et al.*, 2005). In our research, we found a statistically significant difference in deletion of *PIK3CA* gene in CM, probably indicating the loss of this gene in the early steps of esophageal carcinogenesis. Overexpression of *TP63* was found in most ESCC (Hibi *et al.*, 2000, 2001; Glickman *et al.*, 2001; Hu *et al.*, 2002). In a immunohistochemistry study, *TP63* protein was found highly expressed in ESCC, dysplasia, and even in histologically normal epithelia of esophagus adjacent to the cancerous tissues (Glickman *et al.*, 2001). We found two CM cases with numerical gain for *PIK3CA*, and two case with gains for *TP63*, but none had significantly higher copy numbers.

Some CM cases were highlighted for deletion of *FHIT* in chromosome 3 and *EGFR* in chromosome 7, however only chromosome 7 loss was statistically significant. Deletion in both *FHIT* alleles results in loss of exons and concomitant failure in the transcript (Druck et al., 1997). These genomic rearrangements alter the mRNA, leading to absence or reduction of Fhit protein, which is associated with the development of several kinds of cancer (Mori et al., 2000; Kuroki et al., 2003). Loss of heterozygosity (LOH) has also been reported in low-grade dysplasia in regions 3p14-p21 as early events in esophageal carcinogenesis (3p14.2). The *FHIT* gene has been described as hypermethylated in 33% to 69.4% of ESCC cases. Its hypermethylation is also significantly correlated with deletion and loss of protein expression in esophageal carcinoma (Druck et al., 1997).

In the present study, 3 cases showed gain for *MYC*, not statistically significant. One mCGH study revealed gains of 8q23~ter, including the *MYC* locus, but amplification of *MYC* was infrequent in ESCC (1/20) by microarray (Arai et al., 2003) corroborating our results. We also verified 10% (4/40) cases with *FGFR1* gain like Ishisuka et al. (2002) who identified 6% (2/32) of primary ESCC amplified in the locus 8p11. *NCOA3* amplification was observed in 4.9% (2/41) of ESCC specimens (Fujita et al., 2003), amplification/gain of *NCOA3* gene was observed in 13% (15/115) of cases, including high-level amplification in 5 cases (4.3%) and low-level gain in 10 cases (8.7%) (Xu et al, 2008), similarly, we observed 2,5% of CM patients with gain for this gene.

The CM cases identified with genomics deletion and gain were, in the most, grade III and IV, although no statistically significant, excepted for the *MYC* and *YES1* genes. Megaesophagus patients have high variety in esophageal microbiota, which consists mainly of Gram-positive anaerobic bacteria, correlated with the dilation grade (Pajecski et al., 2002; Gockel et al., 2006). These data suggest that the level of dilation and inflammation of megaesophagus could promote genetic damages and

could be related to increased risk of tumor development (Chino et al., 2000; Bektas et al., 2001; Lehman et al., 2001).

Numerical alterations of chromosomes 7, 11 and 17 and *TP53* deletion, by FISH technique, have been reported in megaesophagus by our research group in previous study (Manoel-Caetano et al., 2004). However, recent subset analyses did not find mutations *CDKN2A* (exons 1 and 2) *FHIT* (exons 5 and 7), suggesting these events are uncommon in CM (Manoel-Caetano et al., 2009). Similar data were found for p16 and Fhit protein expression, with no significant decrease in the level of protein expression in these patients (Bellini et al., 2008).

In conclusion, despite the relevance of genomic imbalances involving *TP63*, *EGFR*, *MYC*, *FGFR1*, *YES1* and *NCOA3* in the esophageal carcinogenesis, these genes were not found unbalanced in chagasic megaesophagus. Only *PIK3CA* and *CDKN2A* showed deletion pattern and losses of chromosomes 7 and 9 could be related to the early process of carcinogenesis in chagasic megaesophagus. These findings suggest that genomic imbalances are not promising biomarkers for assessment of ESCC risk in CM.

Acknowledgements

The authors are grateful to Dr. Kenji Miyazaki and Henrique Oliveira for collected the biopsies; Dr. Patricia Maluf Cury for supply the paraffin embedded material; Dr. Sebastião Roberto Taboga and Luiz Roberto Faleiros for help with histological sections. We also thank Brazilian agency CAPES and UCCC Cancer Center of University of Colorado for financial and technical support.

References

Bektas A, Yasa MH, Kuzu I, Dogan I, Unal S, Ormeci N (2001) Flow Cytometric DNA analysis and immunohistochemical p53, PCNA and

histopathologic study in primary achalasia: preliminary results.
Hepatogastroenterology 48:408-412.

Bellini MF, Leite KRM, Cury PM, Silva AE (2008) p53, p16 and Fhit proteins expressions in chronic esophagitis and chagas disease.
Anticancer Res. 28(6B):3793-3799.

Bizari L, Borim AA, Leite KR, Gonçalves F de T, Cury PM, Tajara EH and Silva AE (2006) Alterations of the CCND1 and HER-2/neu (ERBB2) proteins in esophageal and gastric cancers. *Cancer Genet Cytogenet.* 165(1):41-50.

Brasileiro Filho, G. (2006) *Bogliolo Patologia*. Guanabara Koogan, 7th ed.

Brücher BLDM, Stein HJ, Bartels H, Feussner H, Siewert JR(2001) Achalasia and esophageal cancer: incidence, prevalence and prognosis.
World J Surg 25:745-749.

Chang MS Lee HS, Lee BL, Kim YT, Lee JS, Kim WH (2005) Differential protein expression between esophageal squamous cell carcinoma and dysplasia, and prognostic significance of protein markers. *Pathol Res Pract.* 201(6):417-425.

Chen PL, Cheng YW, Chiang CC, Tseng SH, Chau PS, Tsai YY (2006) Hypermethylation of the p16 gene promoter in pterygia and its association with the expression of DNA methyltransferase 3b, *Molecular Vision* 12:1411-1416.

Chino O, Kijima H, Shimada H, Nishi T, Tanaka H, Oshiba G et al (2000) Clinicopathological studies of esophageal carcinoma in achalasia: analysis of carcinogenesis using histological and immunohistochemical procedures.
Anticancer Res 20:3717-3722.

Crawford JM (2004) *The Gastrointestinal Tract*. In: Cotran RS, Kumar V and Collins T *Pathologic basis of disease*. Philadelphia: WB Saunders Company 7th ed.

Daigo Y and Nakamura Y (2008) From cancer genomics to thoracic oncology: discovery of new biomarkers and therapeutic targets for lung and esophageal carcinoma. *Gen Thorac Cardiovasc Surg.* 56(2):43-53.
D'Amico TA (2006) Molecular biologic staging of esophageal cancer. *Thorac Surg Clin.* 16(4):31-327

de Rezende J, Lauar KM, de Oliveira A (1960) Clinical and radiological aspects of aperistalsis of the esophagus. *Rev Bras Gastroenterol.* 12:247-262.

Druck T, Hadaczek P, Fu TB, Ohta M, Siprashvili Z, Baffa R et al (1997) Structure and expression of the human FHIT gene in normal and tumor cells. *Cancer Res.* 57:504-512.

Fujita Y, Sakakura C, Shimomura K, Nakanishi M, Yasuoka R, Aragane H et al. (2003) Chromosome arm 20q gains and other genomic alterations in esophageal squamous cell carcinoma, as analyzed by comparative genomic hybridization and fluorescence in situ hybridization. *Hepatogastroenterol.* 50(54):1857-1863.

Fujiwara S, Noguchi T, Takeno S, Kimura Y, Fumoto S, Kawahara K (2008) Hypermethylation of p16 gene promoter correlates with loss of p16 expression that results in poorer prognosis in esophageal squamous cell carcinomas. *Dis of the Esophagus* 21:125–131.

Fukuoka T, Hibi K, Nakao A (2006) Aberrant methylation is frequently observed in advanced esophageal squamous cell carcinoma. *Anticancer Res.* 26(5A):3333-3335.

Glickman JN, Yang A, Shahsafaei A, McKeon F, Odze RD (2001) Expression of p53-related protein p63 in the gastrointestinal tract and in esophageal metaplastic and neoplastic disorders. *Hum Pathol.* 32(11):1157-1165

Gockel I, Kammerer P, Brieger J, Heinrich UR, Mann WJ, Bittinger F et al (2006) Image cytometric DNA analysis of mucosal biopsies in patients with primary achalasia. *World J Gastroenterol.* 21(12 -19):3020-3025.

Guo M, Ren J, House MG, Qi Y, Brock MV, Herman JG (2006) Accumulation of promoter methylation suggests epigenetic progression in squamous cell carcinoma of the esophagus. *Clin Cancer Res.* 12(15):4515-4522.

Guo XQ, Wang SJ, Zhang LW, Wang XL, Zhang JH, Guo W (2007) DNA methylation and loss of protein expression in esophageal squamous cell carcinogenesis of high-risk area. *J Exp Clin Cancer Res.* 26(4):587-594.

Hanawa M, Suzuki S, Dobashi Y, Yamane T, Kono K, Enomoto N et al (2006) EGFR protein overexpression and gene amplification in squamous cell carcinomas of the esophagus. *Int J Cancer.* 118(5):1173-1180.

Haubrich WS, Schaffner F, Berk JE (1995) Gastroenterology. *Philadelphia: WB Saunders Company* 5th ed.

Hibi K, Trink B, Patturajan M, Westra WH, Caballero OL, Hill DE et al (2000) AIS is an oncogene amplified in squamous cell carcinoma. *Proc Natl Acad Sci* 97(10):5462-5467.

Hibi K, Nakayama H, Taguchi M, Kasai Y, Ito K, Akiyama S et al (2001) AIS overexpression in advanced esophageal cancer. *Clin Cancer Res.* 7(3):469-472.

Hu H, Xia SH, Li AD, Xu X, Cai Y, Han YL et al (2002) Elevated expression of p63 protein in human esophageal squamous cell carcinomas. *Int J Cancer* 102: 580-583.

Ito S, Ohga T, Saeki H, Watanabe M, Kakeji Y, Morita M et al. Promoter hypermethylation and quantitative expression analysis of CDKN2A (p14ARF and p16INK4a) gene in esophageal squamous cell carcinoma. *Anticancer Res.* 27(5A):3345-3353, 2007.

Kato H, Yoshikawa M, Fukai Y, Tajima K, Masuda N, Tsukada K et al (2000) An immunohistochemical study of p16, pRb, p21 and p53 proteins in human esophageal cancers. *Anticancer Res.* 20:345-349.

Kraichely RE, Farrugia G (2006) Achalasia: physiology and etiopathogenesis. *Diseases of the esophagus* 19:213-223.

Kuroki T, Trapasso F, Yendamuri S, Matsuyama A, Alder H, Mori M et al (2003) Allele loss and promoter hypermethylation of VLH, RAR-beta, RASSF1A, and FHIT tumor suppressor genes on chromosome 3p in esophageal squamous cell carcinoma. *Cancer Res.* 63:3724-3728.

Kuwano H, Kato H, Miyazaki T, Fukuchi M, Masuda N, Nakajima M et al (2005) Genetic alterations in esophageal cancer. *Surg Today* 35:7-18.

Lana M de, Tafuri WL (2000) In: Neves DP *Parasitologia Humana*. São Paulo: Atheneu, 10th ed.

Lehman MB, Clark SB, Ormsby AH, Rice TW, Richter JE, Goldblum JR (2001) Squamous mucosal alterations in esophagectomy specimens from patients with end-stage achalasia. *Am J Surg Pathol* 25:1413-1418.

Li L and Yang T (2003) Expression of Cdk4, cyclin D1 and Rb in exogenous wild type P16 gene transfected bladder cancer cell line. *Colloids and Surfaces B: Biointerfaces* 30:315–322.

Lo HW and Hung MC (2006) Nuclear EGFR signalling network in cancers: linking EGFR pathway to cell cycle progression, nitric oxide pathway and patient survival. *British Journal of Cancer* 94, 184 – 188.

Manoel-Caetano F da S, Borim AA, Caetano A, Cury PM, Silva AE (2004) Cytogenetic alterations in chagasic achalasia compared to esophageal carcinoma. *Cancer Genet Cytogenet.* 149(1):17-22.

Manoel-Caetano FS, Silveira AFP, Silva AE (2009) Gene mutations in esophageal mucosa of Chagas disease patients. *Anticancer Res*, *in press*.

Matsuo K, Hamajima N, Shinoda M, Hatooka S, Inoue M, Takezaki T et al (2001) Gene-environment interaction between an aldehyde dehydrogenase-2 (ALDH2) polymorphism and alcohol consumption for the risk of esophageal cancer. *Carcinogenesis* 22:913-916.

Mimura K, Kono K, Hanawa M, Mitsui F, Sugai H, Miyagawa N et al (2005) Frequencies of HER-2/neu expression and gene amplification in patients with oesophageal squamous cell carcinoma. *Br J Cancer* 92(7):1253-60.

Mori T, Miura K, Aoki T, Nishihira T, Mori S, Nakamura Y (1994) Frequent somatic mutation of the MTS1/CDK4I (multiple tumor suppressor/cyclin-dependent kinase 4 inhibitor) gene in esophageal squamous cell carcinoma. *Cancer Res.* 54:3396-3397.

Mori M, Mimori K, Shiraishi T, Alder H, Inoue H, Tanaka Y et al. Altered expression of Fhit in carcinoma and precarcinomatous lesions of the esophagus. *Cancer Res.* 60:1177-1182.

Nuovo G, Plaia TW, Belinsky SA, Baylin SB, Herman JG (1999) *In situ* detection of the hypermethylation-induced inactivation of the *p16* gene as an early event in oncogenesis. *PNAS*, 96(22):12754-12759.

Pajcecki D, Zilberstein B, dos Santos MA, Ubriaco JA, Quintanilha AG, Cecconello I et al (2002). Megaesophagus Microbiota: A Qualitative and Quantitative Analysis. *Journal of Gastrointestinal Surgery* 6:723 – 729.

Pelucchi C, Galeone C, Montella M, Polesel J, Crispo A, Talamini R et al (2008) Alcohol and tobacco use, and cancer risk for upper aerodigestive tract and liver. *Eur J Cancer Prev.* 17(4):340-344.

Pimkhaokham A, Shimada Y, Fukuda Y, Kurihara N, Imoto I, Yang ZQ et al (2000) Nonrandom Chromosomal Imbalances in Esophageal Squamous Cell Carcinoma Cell Lines: Possible Involvement of the *ATF3* and *CENPF* Genes in the 1q32 Amplico Jpn J Cancer Res. 91:1126–1133.

Pinotti, HW (1996) In: Coelho JCU Aparelho Digestivo. Rio de Janeiro: MDESI, 2th ed.

Psyrris A, Yu Z, Weinberger PM, Sasaki C, Haffty B, Camp R et al (2005) Quantitative determination of nuclear and cytoplasmic epidermal growth factor receptor expression in oropharyngeal squamous cell cancer by using automated quantitative analysis. Clin Cancer Res 11: 5856–5862

Ralhan R, Mathew R, Arora S, Bahl R, Shukla NK, Mathur M (2000) Frequent alterations in the expression of tumor suppressor genes p16INK4A and pRb in esophageal squamous cell carcinoma in the Indian population. J. Cancer Res. Clin. Oncol. 126:655-660.

Roth MJ, Abnet CC, Hu N, Wang QH, Wei WQ, Green L et al (2006) p16, MGMT, RARbeta2, CLDN3, CRBP and MT1G gene methylation in esophageal squamous cell carcinoma and its precursor lesions. Oncol Rep. 15(6):1591-1597.

Safatle-Ribeiro AV, Ribeiro U Jr, Sakai P, Clarke MR, Fylyk SN, Ishioka et al (2000) Integrated p53 histopathologic/genetic analysis of premalignant lesions of the esophagus. Cancer Detect Prev. 24:13-23.

Sunpaweravong P, Sunpaweravong S, Puttawibul P, Mitarnun W, Zeng C, Barón AE et al (2005) Epidermal growth factor receptor and cyclin D1 are independently amplified and overexpressed in esophageal squamous cell carcinoma. J Cancer Res Clin Oncol. 131(2):111-119.

Takeuchi H, Ozawa S, Ando N, Kitagawa Y, Mukai M, Ueda M et al. (2001) Further evidence that altered p16/CDKN2 gene expression is associated with lymph node metastasis in squamous cell carcinoma of the esophagus. Oncol. Rep. 8:627-632.

Xu FP, Liu YH, Zhuang HG, Li L, Luo XL, Wen JM (2008) Role of AIB1 gene in development of esophageal squamous cell carcinoma and its clinicopathologic significance. Zhonghua Bing Li Xue Za Zhi. 37(4):250-254.

Wang J, Sasco AJ, Fu C, Xue H, Guo G, Hua Z, et al. (2008) Aberrant

DNA methylation of P16, MGMT, and hMLH1 genes in combination with MTHFR C677T genetic polymorphism in esophageal squamous cell carcinoma. *Cancer Epidemiol Biomarkers Prev.* 17(1):118-125.

Yen CC, Chen YJ, Pan CC, Lu KS, Chen PCH, Hsia JY et al (2005) Copy number changes of target genes in chromosome 3q25.3-qter of esophageal squamous cell carcinoma: TP63 is amplified in early carcinogenesis but down-regulated as disease progressed. *World J Gastroenterol* 11(9):1267-1272.

Yoshida K, Yasui W, Ito H, Tahara E (1990) Growth factors in progression of human esophageal and gastric carcinomas. *Exp Pathol.* 40(4):291-300.

Internet References

American Cancer Society (2008) Internet Reference. Retrieved from <http://www.cancer.org>. December 8th, 2008.

Graphpad, (2008) Internet Reference. Retrieved from <http://www.graphpad.com/instat>. April 3rd, 2008.

Health A to Z (2008) Internet Reference. Retrieved from http://www.healthatoz.com/healthatoz/Atoz/common/standard/transform.jsp?requestURI=/healthatoz/Atoz/ency/esophageal_cancer.jsp. December 8th 2008.

INCA, Instituto Nacional do Câncer Internet Reference. Retrieved from <http://www.inca.org.br>. December 8th, 2008.

Table 1. Mean copy number per cell and standard deviation (SD) for the 12 DNA targets tested in esophageal mucosa, including 9 genes and 3 centromeric sequences.

Groups	N	Targets (Average±SD)										Centromere	Centromere	Centromere	Centromere
		FHIT	PIK3CA	TP63	EGFR	FGFR1	MYC	CDKN2A	YES1	NCOA3	CEN3				
		3p14.2	3q26.32	3q28	7p12	8p12	8q24	9p21	18p11.31	20q12	Centromere 3				
CM	40														
S - Layer		1.77±0.08	1.76±0.09	1.77±0.11	1.52±0.12 ^a	1.78±0.01	1.76±0.09	1.49±0.08	1.67±0.13	1.65±0.11	1.77±0.08	1.53±0.12	1.53±0.09		
B - Layer		1.78±0.08	1.78±0.08	1.78±0.09	1.45±0.15 ^b	1.76±0.10	1.75±0.09	1.50±0.07	1.67±0.12	1.64±0.11	1.79±0.07	1.50±0.16	1.53±0.09		
Total		1.76±0.08	1.77±0.07	1.77±0.09	1.49±0.13 ^{a,b}	1.77±0.08	1.76±0.08	1.50±0.06	1.67±0.11	1.64±0.10	1.77±0.07	1.52±0.13	1.53±0.07		
Range		1.56-1.90	1.56-1.96	1.56-2.06	1.25-1.80	1.56-2.22	1.58-1.77	1.34-1.59	1.37-1.99	1.37-1.80	1.56-1.90	1.26-1.53	1.33-1.65		
NM															
S - Layer		1.35±0.49	1.70±0.09	1.70±0.09	1.60±0.00	1.63±0.01	1.66±0.01	1.76±0.14	1.70±0.08	1.66±0.11	1.71±0.07	1.67±0.01	1.77±0.15		
B - Layer		1.75±0.04	1.78±0.11	1.78±0.11	1.74±0.01	1.70±0.03	1.74±0.02	1.72±0.01	1.68±0.13	1.61±0.01	1.76±0.08	1.79±0.04	1.77±0.04		
Total		1.74±0.04	1.74±0.01	1.74±0.01	1.67±0.01	1.67±0.03	1.70±0.01	1.74±0.07	1.69±0.07	1.64±0.06	1.74±0.08	1.73±0.21	1.77±0.10		
Range		1.71-1.77	1.64-1.86	1.64-1.86	1.60-1.75	1.62-1.72	1.69-1.70	1.69-1.79	1.58-1.75	1.59-1.68	1.68-1.79	1.76-1.82	1.70-1.84		

CM, Chagasic Megaesophagus; NM, health individuals; SD, standard-deviation; S, superficial; B, basal; Statistical Analysis: *t*-Student ($p<0.05$); ^{a,b}, $p=0.008$.

Table 2. Comparison among the chagasic megaesophagus (CM) and normal mucosa groups based on total counting results for the targets

Targets	CM	NM	Statistical Analysis
	Average $\pm$ SD		
<i>FHIT</i>	1.77 $\pm$ 0.08	1.73 $\pm$ 0.22	ns
<i>PIK3CA</i>	1.77 $\pm$ 0.07	1.74 $\pm$ 0.02	ns
<i>TP63</i>	1.77 $\pm$ 0.09	1.74 $\pm$ 0.01	ns
<i>EGFR</i>	1.49 $\pm$ 0.12	1.67 $\pm$ 0.01	ns
<i>FGFR1</i>	1.77 $\pm$ 0.08	1.67 $\pm$ 0.01	ns
<i>CDKN2A</i>	1.50 $\pm$ 0.08	1.74 $\pm$ 0.07	* p<0.0001
<i>MYC</i>	1.75 $\pm$ 0.07	1.70 $\pm$ 0.01	ns
<i>YES-1</i>	1.67 $\pm$ 0.11	1.69 $\pm$ 0.08	ns
<i>NCOA-3</i>	1.64 $\pm$ 0.10	1.64 $\pm$ 0.06	ns
CEN3	1.77 $\pm$ 0.08	1.74 $\pm$ 0.08	ns
CEP7	1.51 $\pm$ 0.13	1.73 $\pm$ 0.02	ns
CEP9	1.53 $\pm$ 0.08	1.77 $\pm$ 0.10	* p=0.0002

SD, standard-deviation; Statistical Analysis: *t*-Student; * p<0.05; ns, p>0.05

Table 3. Comparison between gene and centromere targets

Gene-Centromere	CM		NM	
	Average $\pm$ SD	Ratio	Average $\pm$ SD	Ratio
FHIT	1.77 $\pm$ 0.08	1.00	1.74 $\pm$ 0.04	1.00
CEN3	1.77 $\pm$ 0.08		1.74 $\pm$ 0.08	
<i>t</i> -Student	ns		ns	
EGFR	1.49 $\pm$ 0.12	0.98	1.67 $\pm$ 0.01	0.96
CEP7	1.51 $\pm$ 0.13		1.73 $\pm$ 0.02	
<i>t</i> -Student	ns		ns	
CDKN2A	1.51 $\pm$ 0.09	1.00	1.74 $\pm$ 0.07	0.98
CEP9	1.54 $\pm$ 0.10		1.77 $\pm$ 0.10	
<i>t</i> -Student	ns		ns	

chagasic megaesophagus, CM; SD, standard-deviation; normal mucosa, NM; NP, assay was not performed; Statistical Analysis: *t*-Student (p<0.05); ns, p>0.05.

Table 4. Mean copy number per cell and ranging of each genomic target in distinct grades of megaesophagus

Targets	Megaesophagus Grade				ANOVA
	I	II	III	IV	
<i>FHIT</i>	1.66 ± 0.331 1.0 - 1.86	1.54±0.319 1.0 - 1.83	1.77±0.067 1.66 - 1.88	1.80±0.080 1.68 - 1.92	p = 0.0810 ^{ns}
<i>PIK3CA</i>	1.75 ± 0.009 1.74 - 1.76	1.78 ± 0.057 1.68 - 1.84	1.77 ± 0.090 1.58 - 1.87	1.77 ± 0.067 1.65 - 1.84	p = 0.9319 ^{ns}
<i>TP63</i>	1.75 ± 0.033 1.71 - 1.79	1.78 ± 0.058 1.68 - 1.83	1.77 ± 0.997 1.58 - 1.89	1.78 ± 0.096 1.65 - 1.99	p = 0.8483 ^{ns}
<i>EGFR</i>	1.50 ± 0.130 1.36 - 1.68	1.51 ± 0.100 1.34 - 1.61	1.49 ± 0.109 1.3 - 1.73	1.47 ± 0.140 1.25 - 1.8	p = 0.9467 ^{ns}
<i>FGFR1</i>	1.77 ± 0.060 1.7 - 1.84	1.81 ± 0.057 1.72 - 1.87	1.76 ± 0.081 1.57 - 1.88	1.73 ± 0.120 1.58 - 2.01	p = 0.3936 ^{ns}
<i>MYC</i>	1.76 ± 0.062 1.68 - 1.85 ^{a,b,c}	1.81 ± 0.058 1.72 - 1.87 ^{a,b}	1.75 ± 0.083 1.6 - 1.89 ^{a,b,c}	1.71 ± 0.070 1.58 - 1.81 ^{a,c}	p = 0.0492 *
<i>CDKN2A</i>	1.50±0.057 1.42 - 1.55	1.48 ± 0.035 1.44 - 1.53	1.49 ± 0.081 1.34 - 1.59	1.51 ± 0.087 1.34 - 1.69	p = 0.8833 ^{ns}
<i>YES-1</i>	1.71 ± 0.088 1.61 - 1.83 ^a	1.64 ± 0.053 1.57 - 1.70 ^{a,b}	1.65 ± 0.088 1.53 - 1.9 ^{a,b}	1.57 ± 0.123 1.37 - 1.78 ^b	p = 0.0463 *
<i>NCOA-3</i>	1.72 ± 0.085 1.61 - 1.83	1.64 ± 0.075 1.53 - 1.73	1.68 ± 0.091 1.54 - 1.91	1.59 ± 0.136 1.37 - 1.8	p = 0.0685 ^{ns}
<i>CEN3</i>	1.80 ± 0.069 1.69 - 1.86	1.73 ± 0.106 1.56 - 1.83	1.77 ± 0.070 1.66 - 1.88	1.79 ± 0.079 1.68 - 1.92	p = 0.3806 ^{ns}
<i>CEP7</i>	1.59 ± 0.129 1.41 - 1.74	1.52 ± 0.115 1.34 - 1.64	1.52 ± 0.097 1.35 - 1.68	1.50 ± 0.153 1.26 - 1.78	p = 0.5688 ^{ns}
<i>CEP9</i>	1.54 ± 0.068 1.45 - 1.62	1.53 ± 0.063 1.44 - 1.62	1.53 ± 0.094 1.33 - 1.63	1.54 ± 0.094 1.34 - 1.69	p = 0.945 ^{ns}

^{ns}, p>0.05; * p < 0.05

Table 5. Frequencies of loss, balanced and gain of the genes in chagasic megasophagus (CM) and health patients (NM).

Megacephalugae (CM) and health patients (NM).								
Target	Loss			Balanced		Gain		
	CM	NM	χ^2 test	CM	NM	CM	NM	
<i>FHIT</i>								
N	20	2	P= 10.672 ^{ns}	2478	144	6	3	
%	0.79	1.34		98.96	96.64	0.24	2.04	
<i>PIK3CA</i>								
N	898	57	P=0.000 ⁺ *	1530	139	35	4	
%	36.39	28.50		61.99	69.50	1.42	2.00	
<i>TP63</i>								
N	885	58	P=2.579 ^{ns}	2878	136	37	6	
%	23.29	29.00		75.74	68.00	0.97	3.00	
<i>EGFR</i>								
N	298	12	P=1.067 ^{ns}	3433	187	171	1	
%	7.59	6.00		88.03	93.50	4.38	0.50	
<i>FGFR1</i>								
N	928	68	P=4.172 ^{ns}	2833	121	46	1	
%	24.38	34.00		74.41	60.50	1.21	0.50	
<i>MYC</i>								
N	918	62	P=4.812 ^{ns}	2849	135	33	2	
%	24.00	31		75.00	67.50	0.01	1.00	
<i>CDKN2A</i>								
N	183	6	P=17,702 ^{ns}	3649	194	68	0	
%	4.69	3.00		93.56	97.00	1.74	0.00	
<i>YES1</i>								
N	1381	62	P=2.171 ^{ns}	2407	136	12	2	
%	36.34	31.00		63.34	68.00	0.32	1.00	
<i>NCOA3</i>								
N	45	8	P= 3,627 ^{ns}	2366	130	8	0	
%	1.86	5.80		97.81	94.20	0.33	0.00	

N, number of cell counted; Statistical Analysis, χ^2 test: *, p<0.05, ^{ns} p>0.05

Table 6. Frequencies of nuclei with aneusomies of 3, 7 and 9 centromeres in the different groups

Centromere	CM		NM		Statistical Analysis
	N	%	N	%	
CEN 3					
< 2 copies	683	21.00	54	27.00	P = 0.470 ^{ns}
2 copies	2510	78.44	145	72.50	
> 2 copies	7	0.56	1	0.50	
CEP 7					
< 2 copies	1637	51.16	56	28.00	P = 0.00 ⁺ *
2 copies	1509	47.16	143	71.50	
> 2 copies	54	1.68	1	0.50	
CEP9					
< 2 copies	1400	43.75	46	23.00	P = 0.00 ⁺ *
2 copies	1468	45.87	154	77.00	
> 2 copies	332	10.38	0	0.00	

CM, Chagasic Megaesophagus; NM, normal mucosa; N number of cells counted; Statistical Analysis, χ^2 test: *, $p < 0.05$, ^{ns} $p > 0.05$.

Legend of figures:

Figure 1. Exploratory analysis using three-dimensional plots of the percentages of loss genes status for individual patients is illustrated. *EGFR*, CM30 ~ 30% of loss; *FHIT*, CM36 ~10% of loss and *YES1*, CM7 ~60% of loss

Figure 2. Exploratory analysis using three-dimensional plots of the percentages of gain and loss genes status for individual patients is illustrated. *PIK3CA*, CM13 and CM19 ~ 40% of loss, CM28 and CM11, between 5 and 10% of gain; *TP63*, CM13 ~ 45% of loss, CM6 and CM7 ~10% of gain; *CDKN2A*, CM12 ~12% of loss, CM 11 and CM 16 ~ 6% of gain; CEP9, CM16, CM34 and CM 36 ~ 60% of loss, CM1 and CM 21 ~ 6% of gain.

Figure 3: Exploratory analysis using three-dimensional plots of the percentages of gain genes status for individual patients is illustrated. *NCOA3*, CM36 ~ 4,5% of gain; *MYC*, CM3, CM6 and CM30 between 5 and 7.5% of gain; *FGFR1*, CM30 ~10% of gain.

Figure 4. FISH images for the unbalanced genes and centromeric targets in the megaesophagus specimens.

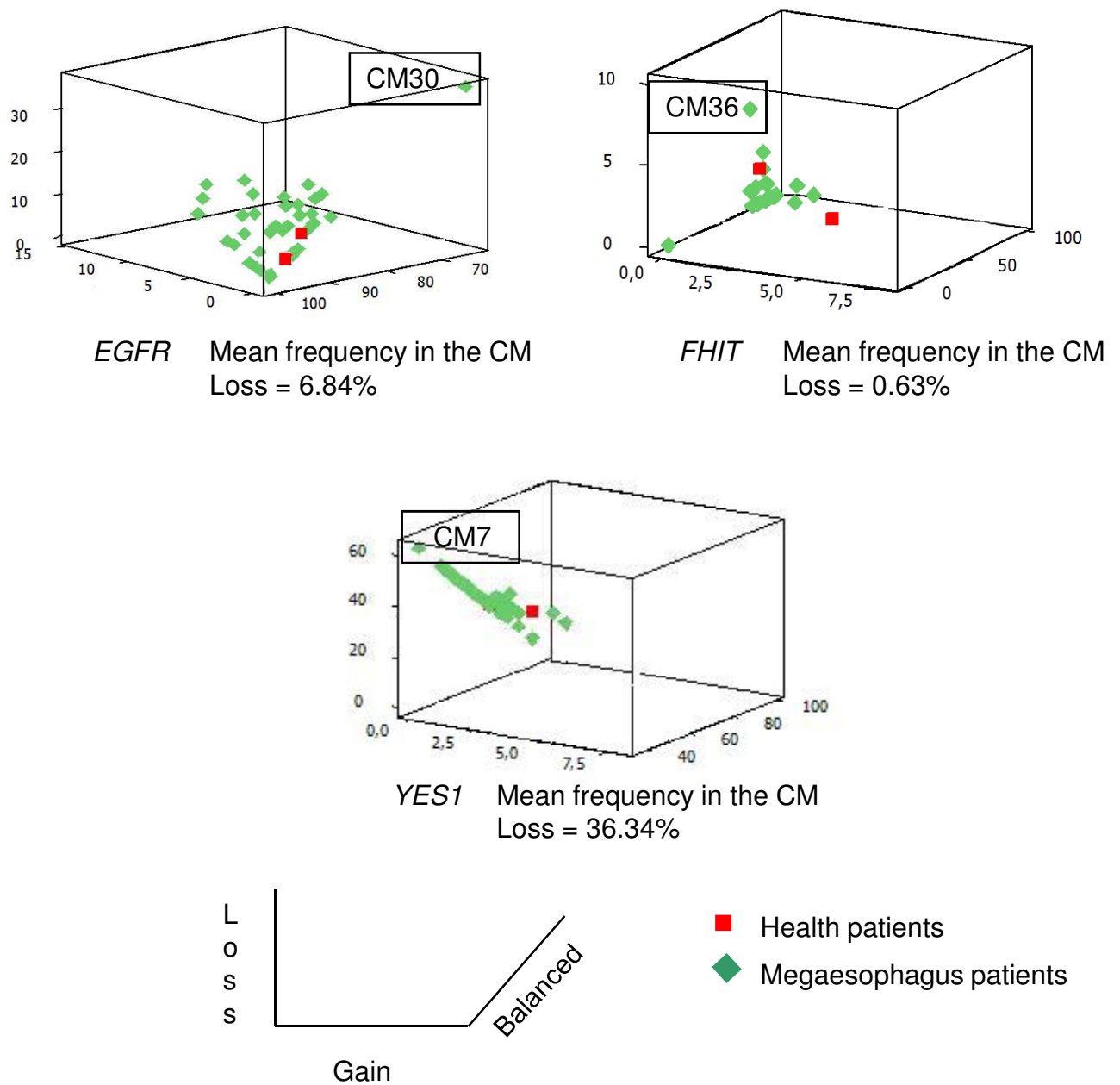
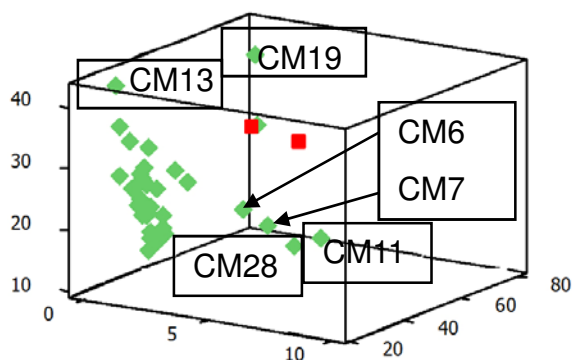
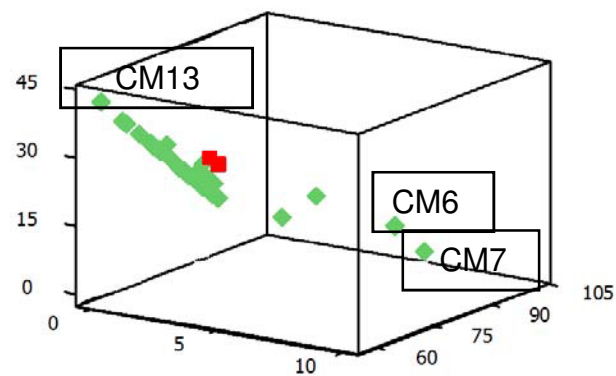


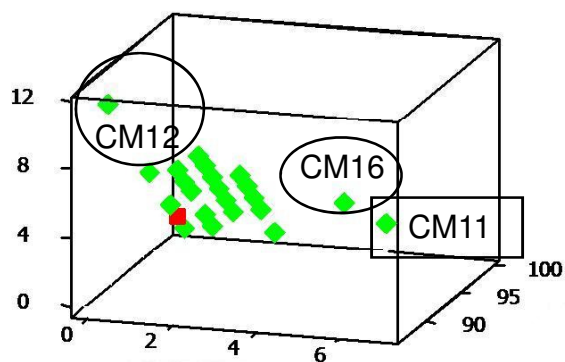
Figure 1.



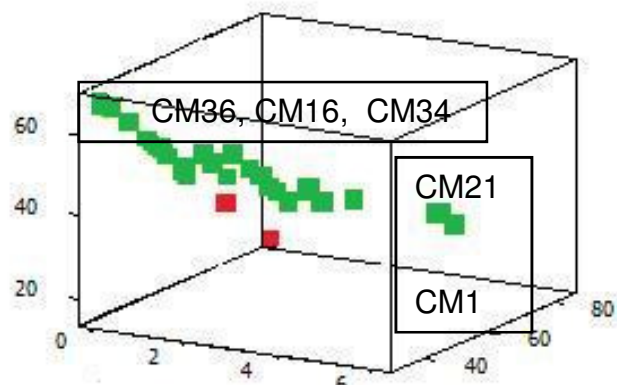
PIK3CA Mean frequency in the CM
Loss: 23.63%
Gain: 0.92%



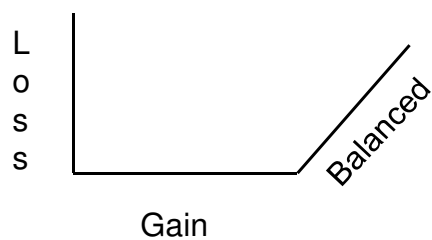
TP63 Mean frequency in the CM
Loss: 23.29%
Gain: 1.07 %



CDKN2A Mean frequency in the CM
Loss: 4.76%
Gain: 1.79%

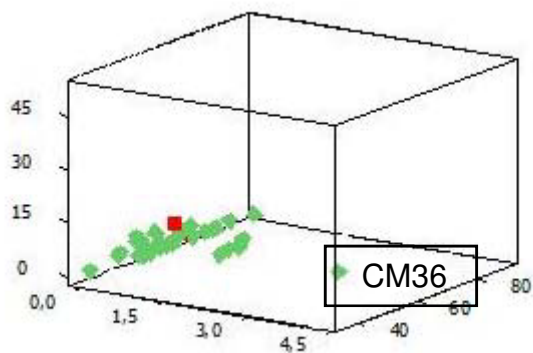


Centromere 9 Mean frequency in the CM
Loss: 46.5%
Gain: 1.21%

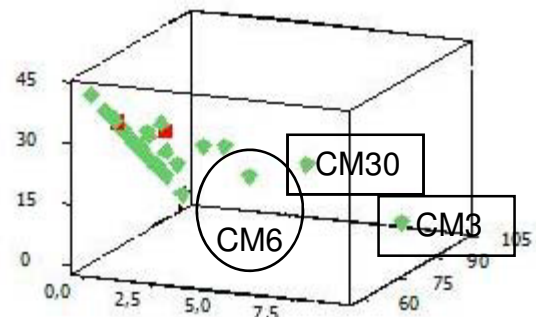


■ Health patients
◆ Megaesophagus patients

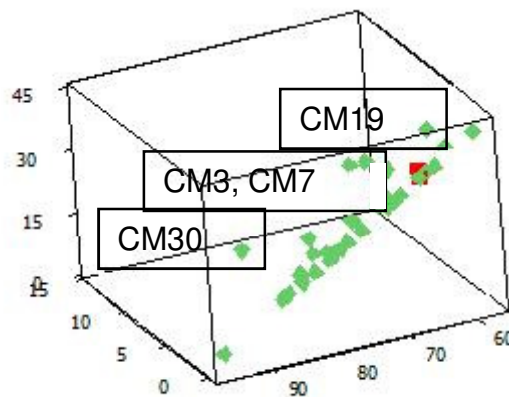
Figure 2.



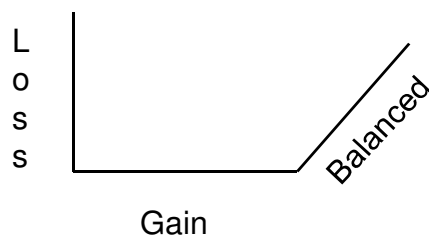
NCOA3 Mean frequency in the CM
Gain = 0.21%



MYC Mean frequency in the CM
Gain = 0.87%

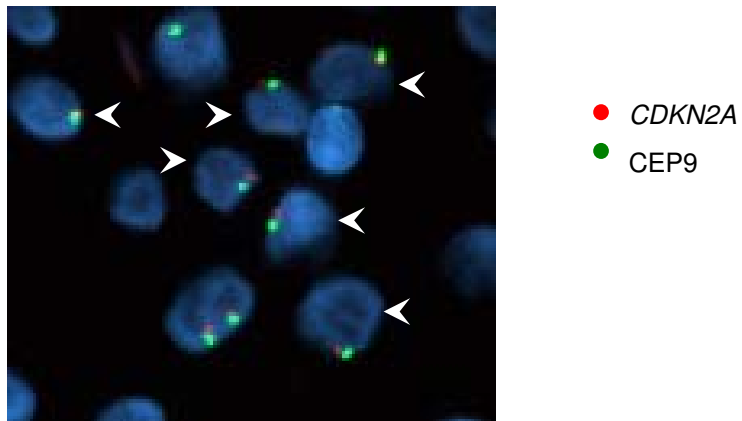


FGFR1 Mean frequency in the CM
Gain = 1.21%

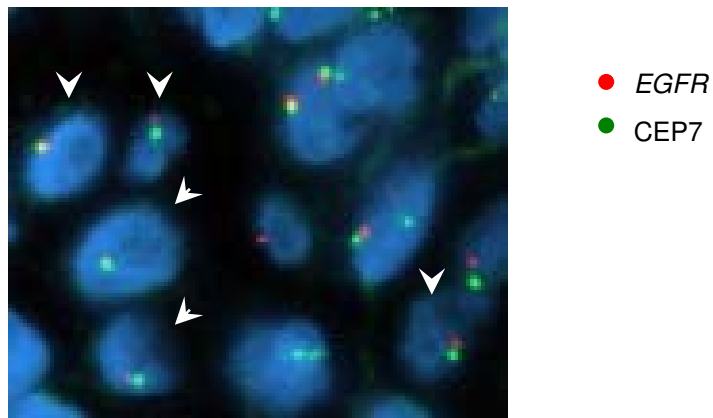


■ Health patients
◆ Megaesophagus patients

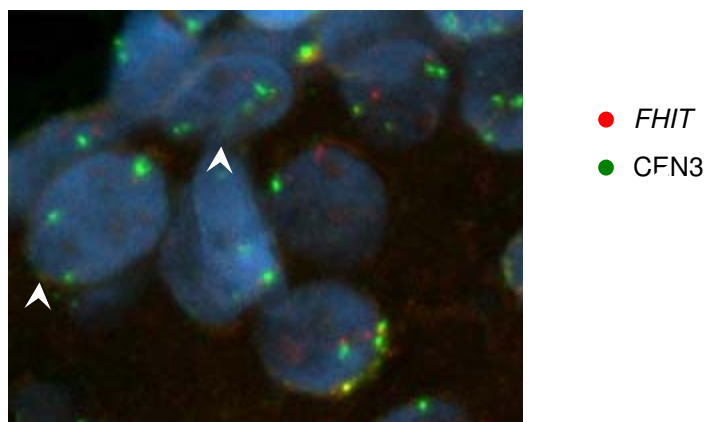
Figure 3.



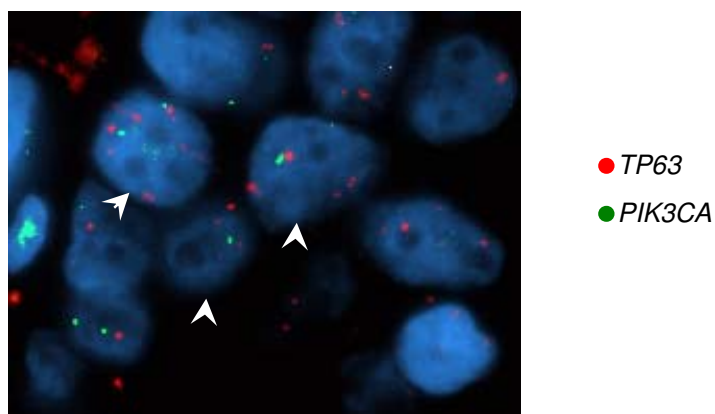
A. Loss *CDKN2A* and centromere 9



B. Loss of *EGFR* and centromere 7



C. Loss *FHIT* and gain of centromere 3



D. Gain of *TP63* and loss of *PIK3CA*

Figure 4.

Discussão

III. DISCUSSÃO

No presente estudo, a avaliação de alterações na expressão de proteínas reguladoras do ciclo celular, como p53, p16 e Fhit, e dos níveis de Ki67 e CPP32, em lesões benignas do esôfago com potencial pré-canceroso mostrou expressão aumentada da proteína p53 com um aumento crescente de casos p53 positivos de acordo com a severidade da lesão (megaesôfago, esofagite e carcinoma). Assim indicando participação desta nas etapas iniciais da carcinogênese do esôfago. Entretanto, para as proteínas p16 e Fhit não foram observadas alterações importantes em suas expressões entre os diferentes grupos. Da mesma forma, também não se observou alterações significantes nos níveis de proliferação celular e apoptose nos grupos de lesões benignas, mas apenas uma frequência maior de casos CPP32 positivos nos carcinomas. Também não foi evidenciada a ocorrência de concordância na expressão alterada das proteínas p53, p16 e Fhit nos grupos avaliados, nem associação da expressão alterada da p53 com a expressão de CPP32 e antígeno Ki67. Nestas avaliações não foi possível estabelecer associações entre essas proteínas e parâmetros como idade, sexo e hábitos tabagista e etilista. O estudo citogenético molecular em pacientes com megaesôfago chagásico, pela técnica FISH, para avaliação de perdas e ganhos de genes freqüentemente em desequilíbrio em carcinoma esofágico (*FHIT*, *TP63*, *PIK3CA*, *EGFR*, *FGFR1*, *MYC*, *CDKN2A*, *YES1*, *NCOA3*, e centrômeros 3, 7 e 9), mostrou que o número de cópias dos genes nas camadas superficial e basal da mucosa esofágica eram estatisticamente diferentes apenas para *EGFR*, com menor número de cópias na camada basal. Neste estudo, observaram-se diferenças significantes no grupo de megaesôfago como perda de *CDKN2A*, *PIK3CA* e

dos cromossomos 7 e 9, associados principalmente aos graus III e IV do megaesôfago, ainda que diferença significativa não tenha sido observada entre o número de cópias para a maioria dos alvos genômicos estudados e os graus de megaesôfago, exceto para os genes *MYC* e *YES1*.

O gene *TP53* desempenha papel importante na regulação do ciclo celular, mas quando alterado é um dos principais genes envolvidos em cerca de 50% das neoplasias humanas (ASKER; WIMAN; SELIVANOVA 1999). No presente estudo pode ser constatado um aumento crescente de casos p53 positivos a partir do grupo de chagásicos sem megaesôfago (7,7%), com megaesôfago (26,1%), esofagite crônica (52,2%) e carcinoma esofágico (100%). Fuji *et al.* (2000), avaliando a natureza pré-maligna de megaesôfago, analisaram a expressão de p53, por imuno-histoquímica, em pacientes com carcinoma esofágico que apresentavam megaesôfago e as regiões de epitélio não maligno com esofagite e/ ou displasia. Os autores verificaram expressão aumentada de p53 nos tumores, mas não no epitélio não-neoplásico, como na esofagite e displasia. Contudo, no epitélio de megaesôfago, observou além de alterações histológicas em vários graus, a expressão aumentada de p53. Da mesma forma, Fagundes *et al.* (2005) também detectaram a imunoexpressão da proteína p53 de forma crescente em indivíduos com mucosa normal (12%), esofagite moderada (22%), esofagite severa (33%), displasia (36%) e carcinoma (100%), com predomínio da expressão em fumantes e etilistas, sugerindo que pacientes que apresentam esofagite crônica, com hiperexpressão de p53 têm risco elevado para desenvolver câncer de esôfago. Entretanto, mais recentemente, Gockel *et al.* (2006), não encontraram positividade de p53 em nenhum dos 15 pacientes com megaesôfago estudados. ,

Os pacientes com megaesôfago chagásico frequentemente apresentam esofagite crônica, devido à retenção do bolo alimentar (estase esofágica) no esôfago dilatado, aumentando o risco de proliferação

bacteriana e danos à mucosa (CHINO *et al.*, 2000; FUJI *et al.*, 2000; PAJECKI *et al.*, 2002). Entre essas bactérias estão caracterizadas aquelas pertencentes a microbiota normal da boca e orofaringe, que são responsáveis por criar um ambiente favorável a microrganismos anaeróbios, capazes de reduzir nitratos em nitritos, gerando carcinógenos, que poderão desencadear o processo de carcinogênese esofágica (PAJECKI *et al.*, 2002). No estudo realizado 69% dos pacientes com megaesôfago também apresentavam esofagite crônica. Portanto, os casos com expressão alterada de p53, (6/23) em megaesôfago chagásico e (12/23) em esofagite crônica, podem ter ocorrido em resposta ao processo inflamatório e/ou a injúrias no DNA, enquanto que em carcinoma de células escamosas de esôfago a expressão acentuada da proteína nos 11 casos estudados deve estar relacionada com mutações no gene *TP53*. A frequência maior de casos p53 positivos no grupo de esofagite crônica reforça a participação do processo inflamatório na expressão dessa proteína. Murphy *et al.* (2005) observaram um aumento moderado no risco de desenvolvimento de carcinoma de esôfago, mas não de adenocarcinoma em pacientes com esofagite, confirmando que a lesão precursora de adenocarcinoma seja o esôfago de Barret e que esofagite possa ser uma lesão precursora de carcinoma de células escamosas do esôfago.

A perda ou redução da expressão da proteína p16 pode estar associada com mutações de ponto, deleções, perda de heterozigose ou processos que inativam a expressão gênica, como a hipermetilação da região promotora (HU *et al.*, 2004; KRÜGER *et al.*, 2005; SANTIN *et al.*, 2005). Em estudo de carcinoma esofágico familiar e esporádico, no Irã, foi observado que 64,3% dos membros da família estudada apresentavam o gene *CDKN2A* (*MTS-1*; *P16*) com hipermetilação na região promotora. Nos casos esporádicos, essa frequência foi maior nas amostras de tecido tumoral, ou seja, 73,3%, mas nas amostras de soro e sangue, dos mesmos pacientes,

a hipermetilação foi de 26,6% e 43,3%, respectivamente (ABBASZADEGAN *et al.*, 2005). Assim, revelando uma forte associação com o processo de hipermetilação do gene apenas no tecido tumoral, e indicando que o gene *CDKN2A* mutante pode ser utilizado como biomarcador na identificação precoce de carcinoma esofágico em populações de alto risco e em casos de história familiar. No presente estudo, entretanto, a análise da expressão da proteína p16 em pacientes com lesões esofágicas benignas e em carcinoma esofágico se mostrou significativamente alterada apenas no grupo de esofagite crônica, visto que foram encontradas perdas na expressão desta proteína em frequência elevada também no grupo de mucosa normal. Entretanto, o estudo citogenético molecular ora realizado mostrou média menor de número de cópias de *CDKN2A* e do centrômero 9 no grupo de megaesôfago, indicando perda desses alvos genômicos nesta lesão.

A expressão anormal da proteína Fhit foi descrita como um evento freqüente no estágio inicial do desenvolvimento do carcinoma de esôfago, devido à perda completa ou redução da expressão, aumentando de acordo com a progressão da severidade das alterações histopatológicas da mucosa, ou seja, de displasia (média, moderada e severa) para carcinoma *in situ* e carcinoma invasivo (MORI *et al.*, 2000; KITAMURA *et al.*, 2001). No presente estudo, essa expressão anormal foi observada principalmente no grupo de esofagite, mas a análise estatística empregada não mostrou diferença entre os grupos estudados. A análise citogenética de perdas e ganhos destacou um caso de megaesôfago com deleção de *FHIT*, embora não tenha sido observado diferenças significantes na média de número de cópias desse gene e centrômero 3 entre os grupos de megaesôfago e mucosa normal.

As alterações necessárias para o surgimento de câncer envolvem vários genes que controlam o crescimento, a proliferação celular e a homeostase do tecido por meio de apoptose (KOCH *et al.*, 1994). Um dos

marcadores de proliferação celular é o antígeno nuclear Ki67 expresso nas fases G₁, S e M do ciclo celular. A expressão aumentada de Ki67 nuclear tem sido associada à progressão de uma variedade de tumores (LUTTGES *et al.*, 2000 apud LEBE *et al.*, 2004). Nos grupos de mucosa normal, chagásicos sem megaesôfago, esofagite crônica e carcinoma 100% dos casos foram classificados como Ki67 positivos, pois apresentaram mais de 10% de positividade para marcação do antígeno. Enquanto que no grupo de megaesôfago 91,3% casos foram positivos para Ki67. Porém, a análise estatística para os casos positivos, não indicou diferenças estatisticamente significativas entre os grupos.

A avaliação de apoptose utilizando a marcação citoplasmática da proteína caspase-3 (CPP32) em lesões primárias de ESCC tem mostrado que 60% dos casos são positivos (HSIA *et al.*, 2003). No presente estudo, a coloração positiva para CPP32 foi observada em poucos casos de mucosa normal 20% (2/10), sendo interpretada como nível normal de apoptose, e em frequências similares entre os grupos CD, CM e CE respectivamente, 30,8% (4/13), 30,4% (7/23) e 34,8% (8/23), mas aumentada em ESCC, 55,5% (5/9), corroborando alguns estudos da literatura sugerindo que este evento esteja relacionado com melhor prognóstico.

A proteína p53 desempenha uma importante função na indução de apoptose em resposta a danos no DNA (LEVINE, 1997; STEELE *et al.*, 1998). As células bloqueadas na fase G₁ permitem o reparo ou induzem a apoptose se houver falhas nesse mecanismo, prevenindo assim, a transmissão de lesões no DNA às células filhas (LANE, 1992, 1994). Nesse contexto, a inativação da proteína p53 pode promover a desregulação do ciclo celular e a inibição de apoptose, fornecendo forte vantagem no crescimento de células tumorais, como evidenciado no presente estudo, em que observamos alteração na expressão da proteína p53 nas lesões

benignas e carcinoma. Entretanto, não foram estabelecidas associações entre a expressão alterada da proteína p53 com o antígeno Ki67 ou a proteína CPP32.

Além de fatores ambientais e patogênicos, alterações genéticas, como aneuploidias, deleções alélicas, ativação de oncogenes e inativação de genes supressores tumorais estão associadas a carcinogênese esofágica (KUWANO *et al.*, 2005; DAIGO; NAKAMURA, 2008; D'AMICO, 2008). Estudos de citogenética molecular, como mCGH, aCGH, SKY e FISH indicam algumas regiões cromossômicas como alvo de alteração durante o desenvolvimento do câncer. Entre elas podemos citar as regiões 1, 2q, 3q, 5p, 6p, 7, 8q, 9q, 11q, 12p, 14q, 15q, 16, 17, 18p, 19q, 20q, 22q e X, para ganhos, com amplificações em 1q32, 2p16-22, 3q25-28, 5p13-15.3, 7p12-22, 7q21-22, 8q23-24.2, 9q34, 10q21, 11p11.2, 11q13, 13q32, 14q13-14, 14q21, 14q31-32, 15q22-26, 17p11.2, 18p11.2-11.3, 20p11.2; e as seguintes regiões envolvidas em perdas 3p, 4, 5q, 6q, 7q, 8p, 9, 10p, 12p, 13, 14p, 15p, 18, 19p, 20, 22, Xp and Y (SHIMADA *et al.*, 1992; PACK *et al.*, 1999; SHINOMIYA *et al.*, 1999; MAYAMA *et al.*, 2000; PIMKHAOKHAM *et al.* 2000; YEN *et al.*, 2001, 2003; KAMITANI *et al.*, 2002; HSIA *et al.*, 2003; KWONG *et al.*, 2004; QIN *et al.*, 2004, 2005ab, 2008; SU *et al.*, 2006; SUGIMOTO *et al.*, 2007; YANG *et al.* 2008). Além dessas regiões, os genes *PIK3CA* (3q26), *TP63* (3p28), *EGFR* (7p11), *YES1* (18p11.31) e *NCOA3* (20q12) foram encontrados amplificados em frequências elevadas, enquanto são encontradas deleções nos genes supressores de tumor *CDKN2A* (9p21), *MTAP* (9p21) and *TP53* (17p13.1) em ESCC (SHIMADA *et al.*, 1992; PIMKHAOKHAM *et al.*, 2000; NAKAKUKI *et al.*, 2002; YEN *et al.*, 2003; XU *et al.*, 2007; CARNEIRO *et al.*, 2008).

No presente estudo, a análise de perdas e ganhos, no grupo de megaesôfago, de genes frequentemente em desequilíbrio em ESCC, mostrou que o número de cópias do gene *EGFR* era menor na camada basal

da mucosa esofágica que na superficial, e um caso de megaesôfago foi destacado para perda desse gene. Outras alterações também foram evidenciadas como uma média menor de número de cópias de *CDKN2A* e centrômero 9, padrão de deleção do gene *PIK3CA* e perda dos cromossomos 7 e 9. Apesar dos demais genes investigados não apresentarem alterações significantes, podemos destacar alguns casos de megaesôfago com ganho em *PIK3CA*, *TP63*, *CDKN2A*, *FGFR1*, *MYC*, *NCOA3* e centrômero 9, ou deleção em *FHIT*, *PIK3CA*, *EGFR*, *CDKN2A*, *TP63*, *YES1* e centrômero 9, sempre associados aos graus III e IV de megaesôfago. Sabe-se que pacientes com megaesôfago apresentam grande variedade na microbiota esofágica, correlacionadas ao grau de dilatação esofágica e processo inflamatório (PAJECKI *et al.*, 2002; GOCKEL *et al.*, 2006). Portanto, a maior incidência de câncer nos pacientes portadores de megaesôfago pode estar relacionada à ocorrência dessas alterações inflamatórias da mucosa, desencadeando alterações genéticas (PINOTTI *et al.*, 1996), como evidenciado neste estudo. Apesar de, até a conclusão deste estudo, as informações dos prontuários médicos dos pacientes com megaesôfago não revelaram nenhum caso que tenha evoluído para carcinoma. Dessa forma, esses achados citogenéticos em megaesôfago, assim como a expressão alterada da proteína p53 tanto em megaesôfago como em esofagite crônica são relevantes, pois evidenciam a ocorrência de alterações genéticas em lesões benignas precursoras como eventos precoces que devem participar das etapas iniciais da carcinogênese do esôfago.

Conclusões

IV. CONCLUSÕES

No presente estudo, considerando-se as amostras avaliadas e as técnicas utilizadas, obtemos as seguintes conclusões:

a) A expressão da proteína p53 é aumentada nas lesões benignas estudadas, obedecendo a uma progressão em relação à severidade histopatológica da lesão, a partir do megaesôfago, esofagite crônica e carcinoma indicando sua participação desde os estágios iniciais até tardios da carcinogênese esofágica;

b) A expressão das proteínas p16 e Fhit não apresentam alterações significantes nos grupos de lesões benignas e carcinomas avaliados, não havendo indicação de que desempenhem um papel importante na progressão das lesões estudadas;

c) Não há concordância no padrão de expressão das proteínas p53, p16 e Fhit nas lesões benignas e carcinoma, entretanto expressão alterada das proteínas p53 e p16, p53 e Fhit, p16 e Fhit podem estar associadas nestas lesões durante o processo de controle do ciclo celular;

d) Não há evidências de alterações nos níveis de expressão do antígeno Ki67 e da proteína caspase-3, indicando ausência alterações significantes nas taxas de proliferação celular e apoptose nas lesões estudadas;

e) Da mesma forma, também não há evidências de associações entre a expressão alterada das proteínas p53, p16, Fhit, CPP32 e antígeno Ki67 com parâmetros como sexo, idade, hábitos tabagista e etilista nas lesões benignas e carcinomas do esôfago avaliados;

f) Apesar da relevância de desequilíbrios gênicos em *TP63*, *EGFR*, *MYC*, *FGFR1*, *YES1* e *NCOA3* em carcinoma de esôfago, estes genes não apresentam padrões de perda ou ganho em megaesôfago chagásico. Assim não devem encontrar-se desbalanceados neste tipo de lesão do esôfago;

g) Apenas os genes *PIK3CA* e *CDKN2A* apresentam padrão de deleção em megaesôfago chagásico, juntamente com perda dos cromossomos 7 e 9, sendo consideradas como alterações iniciais do processo carcinogênico do esôfago.

h) A observação de que regiões genômicas com desequilíbrios na carcinogênese esofágica não são significativamente afetadas em megaesôfago chagásico sugere que não será possível utilizar tais características como marcadores eficazes de risco para desenvolvimento de carcinoma em megaesôfago.

*Referências
Bibliográficas*

REFERÊNCIAS BIBLIOGRÁFICAS

ABBASZADEGAN, M.R. *et al.* Aberrant p16 methylation, a possible epigenetic risk factor in familial esophageal squamous cell carcinoma. *Int J Gastrointest Cancer*, v. 36, n. 1, p. 47-54, 2005.

ARAI, H. *et al.* Detection of amplified oncogenes by genome DNA microarrays in human primary esophageal squamous cell carcinoma: comparison with conventional comparative genomic hybridization analysis. *Cancer Genet Cytogenet.* v. 146, p.16–21. 2003

ASKER, C.; WIMAN, K.G.; SELIVANOVA, G. p53-induced apoptosis as a safeguard against cancer. *Biochemical and Biophysical Research Communications*, v. 265, p. 1- 6, 1999.

BEKTAS, A. *et al.* Flow Cytometric DNA analysis and immunohistochemical p53, PCNA and histopathologic study in primary achalasia: preliminary results. *Hepatogastroenterology*, v.48, p.408-412, 2001.

BENCHIMOL, S. *et al.* Transformation associated p53 is encoded by a gene on human chromosome 17. *Som. Cell Mol. Gen.*, v.11, p. 505 – 509, 1985.

BLANCHARD, C. *et al.* Eotaxin-3 and uniquely conserved gene-expression profile in eosinophilic esophagitis. *The Journal of Clinical Investigation*, v. 116, n. 2, p. 536-547, 2006.

BLANKENBERG, FG; TAIT, JF; STRAUSS, W. Apoptotic cell death: its implications for imaging in the next millennium. *Eur. J. Nucl. Med.*, v.27, p. 359 - 367, 2000.

BRASILEIRO-FILHO, G. **Bogliolo Patologia**. 7ª ed. Rio de Janeiro: Guanabara Koogan, 2006. 1488 p. ISBN: 8527712229

BRETON, J. *et al.* Simultaneous use of DGGE and DHPLC to screen *TP53* mutations in carcens of the esophagus and cardia from a European

high incidence area (Lower Normandy, France). **Mutagenesis**, v. 18, p.299-306, 2003.

BRÜCHER, B.L.D.M. *et al.* Achalasia and esophageal cancer: incidence, prevalence and prognosis. **World J Surg**, v.25, p.745-749, 2001.

CADWELL, C.; ZAMBETTI, G.P. The effects of wild-type p53 tumor suppressor activity and mutant p53 gain-of-function on cell growth. **Gene**, v. 277, p.15-30, 2001.

CAI, H.Y. *et al.* Changes of serum p53 antibodies and clinical significance of radiotherapy for esophageal squamous cell carcinoma. **World J Gastroenterol**. v. 14, n. 25, p.4082-4086, 2008.

CARNEIRO, A. *et al.* Prognostic impact of array-based genomic profiles in esophageal squamous cell cancer. **BMC Cancer**, v. 8, n. 98, p. 1-19, 2008.

CASTELLETTO, R. *et al.* Pre-cancerous lesions of the oesophagus in Argentina: prevalence and association with tobacco and alcohol. **Int J Cancer**, v. 51, p. 34-37, 1992.

CHAN, W.C. *et al.* p16 tumor suppressor gene mutations in Chinese esophageal carcinomas in Hong Kong. **Cancer Letters**, v. 115, p. 201-206, 1997.

CHANG, M.S. *et al.* Differential protein expression between esophageal squamous cell carcinoma and dysplasia, and prognostic significance of protein markers. **Pathol Res Pract**, v. 201, n. 6, p. 417-425, 2005.

CHEN, Y.J. *et al.* Aberrant FHIT transcripts is cancerous and corresponding non-cancerous lesions of the digestive tract. **Int J Cancer**, v.72, p.955-958, 1997.

CHEN, P.L. *et al.* Hypermethylation of the *p16* gene promoter in pterygia and its association with the expression of DNA methyltransferase 3b, **Molecular Vision**, v.12, p. 1411-1416, 2006.

CHINO, O. *et al.* Clinicopathological studies of esophageal carcinoma in achalasia: analysis of carcinogenesis using histological and immunohistochemical procedures. **Anticancer Res**, v.20, p.3717-3722, 2000.

CHINO, O. *et al.* Accumulation of p53 in esophageal squamous cell carcinoma. **Int J Mol Med**, v.8, p.359-363, 2001.

CHIPUK, J.E.; GREEN, D.R. How do BCL-2 proteins induce mitochondrial outer membrane permeabilization? **Trends Cell Biol.**, v. 18, n. 4, p. 157-164, 2008.

COGGI, G. *et al.* p53 protein accumulation and p53 gene mutation in esophageal carcinoma. **Cancer**, v.79, p.425-432, 1997.

CRAWFORD, J.M. The Gastrointestinal Tract. *In*: COTRAN, RS; KUMAR, V; COLLINS, T. **Pathologic basis of disease**. 7thed Philadelphia: WB Saunders Company, 2004. cap.17, p. 775 – 787.

CUNHA-NETO, E. *et al.* Restricted heterogeneity of T cell receptor variable alpha chain transcripts in heart of Chagas' disease cardiomyopathy patients. **Parasit. Immunology**, v.16, p.171-179, 1994.

CUNHA-NETO, E. *et al.* Autoimmunity in Chagas disease cardiopathy: Biological relevance of cardiac myosin-specific epitope cross-reactive to an immunodominant *Trypanosoma cruzi* antigen. **Proc Nat Acad Sci**, v.92, p.3541-3545, 1995.

DAIGO, Y.; NAKAMURA, Y. From cancer genomics to thoracic oncology: discovery of new biomarkers and therapeutic targets for lung and esophageal carcinoma. **Gen Thorac Cardiovasc Surg**, v. 56, n. 2, p.43-53, 2008.

D'AMICO, T.A. Molecular biologic staging of esophageal cancer. **Thorac Surg Clin**, v.16, n.4, p.317-327, 2006.

de REZENDE, J.; LAUAR K.M.; de OLIVEIRA, A. Clinical and radiological aspects of aperistalsis of the esophagus. **Rev Bras Gastroenterol**, v. 12, p. 247-262, 1960.

DLAMINI, Z. *et al.* Genealogy, expression and molecular mechanisms in apoptosis. **Pharmacology and Therapeutics**, v. 101, p. 1 – 15, 2004.

DRUCK, T. *et al.* Structure and expression of the human FHIT gene in normal and tumor cells. **Cancer Res**, v.57, p.504-512, 1997.

DU PLESSIS, L. *et al.* Mapping of novel regions of DNA gain and loss by comparative genomic hybridization in esophageal carcinoma in the Black and Colored populations of South Africa. **Cancer Res**, v.59, v. 8, p.1877-1883,1999.

EGASHIRA, A. *et al.* p53 gene mutations in esophageal squamous cell carcinoma and their relevance to etiology and pathogenesis: results in Japan and comparisons with other countries. **Cancer Sci.**, v. 98, n.8, p.1152-1156, 2007.

FAGUNDES, R.B. *et al.* p53 immunoexpression: An aid to conventional methods in the screening of precursor lesions of squamous esophageal cancer in patients at high-risk? **Cancer Detection and Prevention**, v.29, n.3, p.227-232, 2005.

FIGUEROA, J.D. *et al.* Cigarette smoking, body mass index, gastro-esophageal reflux disease, and non-steroidal anti-inflammatory drug use and risk of subtypes of esophageal and gastric cancers by P53 overexpression. **Cancer Causes Control**, *in press*, 2008.

FUJI, T. *et al.* Histopathological analysis of non-malignant and malignant epithelium in achalasia of the esophagus. **Dis Esophagus**, v.13, n. 2, p.110–116, 2000.

FUJITA, Y. *et al.* Chromosome arm 20q gains and other genomic alterations in esophageal squamous cell carcinoma, as analyzed by comparative genomic hybridization and fluorescence in situ hybridization. **Hepatogastroenterol**. v. 50, n. 54, p. 1857-1863, 2003.

FUJIWARA, S. *et al.* Hypermethylation of p16 gene promoter correlates with loss of p16 expression that results in poorer prognosis in esophageal squamous cell carcinomas. **Dis Esophagus**, v. 21, p.125–131, 2008.

FUKUOKA, T.; HIBI, K.; NAKAO, A. Aberrant methylation is frequently observed in advanced esophageal squamous cell carcinoma. **Anticancer Res**, v. 26, n. 5A, p. 3333-3335, 2006.

GOCHHAIT, S. *et al.* Concomitant presence of mutations in mitochondrial genome and p53 in cancer development - a study in north Indian sporadic breast and esophageal cancer patients. **Int J Cancer**, v. 123, n. 11, p. 2580-2586, 2008.

GOCKEL, I. *et al.* Image cytometric DNA analysis of mucosal biopsies in patients with primary achalasia. **World J Gastroenterol**, v.12, n.19, p. 3020-3025, 2006.

GUO, M. *et al.* Accumulation of promoter methylation suggests epigenetic progression in squamous cell carcinoma of the esophagus. **Clin Cancer Res**, v. 12, n. 15, p. 4515-4522, 2006.

GUO, X.Q. *et al.* DNA methylation and loss of protein expression in esophageal squamous cell carcinogenesis of high-risk area. **J Exp Clin Cancer Res**, v. 26, n. 4, p.587-594, 2007.

HAINAUT, P. *et al.* IARC Database of p53 gene mutations in human tumors and cell lines: updated compilation, revised formats and new visualisation tools. **Nucleic Acids Research**, v.26, n.1, p. 205-213, 1998.

HANAWA, M. *et al.* EGFR protein overexpression and gene amplification in squamous cell carcinomas of the esophagus. **Int J Cancer**, v. 118, n. 5, p.1173-1180, 2006.

HAUBRICH, W.S.; SCHAFFNER, F.; BERK, J.E. **Gastroenterology**. 5th.ed. Philadelphia: WB Saunders Company, 1995. 881p. ISBN: 13: 9780721637228

Health A to Z: Disponível em: http://www.healthatoz.com/healthatoz/Atoz/common/standard/transform.jsp?requestURI=/healthatoz/Atoz/ency/esophageal_cancer.jsp. Acesso em 11 de dezembro de 2008.

HIBI, K. *et al.* Molecular detection of p16 promoter methylation in the serum of patients with esophageal squamous cell carcinoma. **Clin Cancer Res**, v.7, p.3135-3138, 2001.

HSIA, J.Y. *et al.* Prognostic significance of caspase-3 expression in primary resected esophageal squamous cell carcinoma. **EJSO**, v. 29, p. 44-48, 2003.

HU, N. *et al.* Frequent inactivation of the TP53 gene in esophageal squamous cell carcinoma from a high-risk population in China. **Clin Cancer Res**, v. 7, p. 883-91, 2001.

HUANG, J.X. *et al.* Relationship between proliferative activity of cancer cells and clinicopathological factors in patients with esophageal squamous cell carcinoma. **World J Gastroenterol**, v. 11, p. 19, p.2956-2959, 2005.

IHC World. Disponível em: http://www.ihcworld.com/protocols/antibodyprotocols/ki67_novocastra.htm. Acesso em 25 de novembro de 2008.

IKEDA, G. *et al.* Prognostic Significance of Biologic Factors in Squamous Cell Carcinoma of the Esophagus. **Cancer**, v. 86, n. 8, p. 1396–1405, 1999.

IMGENEX, Inovation in Functional Genomics. Disponível em: http://www.imgenex.com/emarketing/081606Livinor_Survivin/apoptosis-pathway6.jpg. Acesso em 11 de dezembro de 2008.

INCA, Instituto Nacional do Câncer. Disponível em: <http://www.inca.org.br>. Acesso em 05 de dezembro de 2008.

IQB, Instituto Químico Biológico. Disponível em:
<http://www.iqb.es/digestivo/galeria/endoscopia>. Acesso em 03 de abril de 2007.

ISHIZUKA, T. *et al.* Gene amplification profiling of esophageal squamous cell carcinoma by DNA array CGH. **Biochem Biophys Res Commun**, v. 296, p. 152 – 155, 2002.

ITO, S. *et al.* Promoter hypermethylation and quantitative expression analysis of CDKN2A (p14ARF and p16INK4a) gene in esophageal squamous cell carcinoma. **Anticancer Res**, v. 27, n. 5A, p.:3345-3353, 2007.

IWATA, T. *et al.* p53 and MIB-1 expression of esophageal carcinoma concomitant with achalasia. **Hepatogastroenterology**, 2007 v. 54, n. 77, p.1430-1432, 2007.

JEONG, S.Y.; SEOL, D.W. The role of mitochondria in apoptosis. **BMB Rep**, v. 41, n. 1, p. 11-22, 2008.

JONES, R. *et al.* Heartburn in patients with gastro-oesophageal reflux disease in Germany and Sweden: a study on patients' burden of disease. **Pharmacoeconomics**, v. 21, n.15, p.1091-1102, 2003.

KALLIONIEMI, A. *et al.* Comparative genome hybridization for molecular cytogenetics analysis of solid tumors. **Science**, v.258, n.5083, p. 818-821, 1992.

KAMB, A. *et al.* A cell cycle regulator potentially involved in genesis of many tumor types. **Science**, v.264, p.436-440, 1994.

KAMITANI, S. *et al.* Intratumoral regional variations in copy number of the chromosomal part revealed by microdissection and combined ploidy and comparative genomic hybridization analyses in esophageal squamous cell carcinoma. **Cancer Genet Cytogenet**, v.132, p.30–35, 2002.

KANEKO, K. *et al.* Study of p53 gene alteration as a biomarker to evaluate the malignant risk of Lugol-unstained lesion with non-dysplasia in the oesophagus. **Br J Cancer**, v.96, n. 3, p. 492-498, 2007.

KASTAN, M.B. *et al.* Participation of p53 protein in the cellular response to DNA damage. **Cancer Res**, v.51, p.6304-6311, 1991.

KATİYAR, S. *et al.* p53 gene mutation and human papillomavirus (HPV) infection in esophageal carcinoma from three different endemic geographic regions of India. **Cancer Letters**, p. 1-11, 2004.

KATO, H. *et al.* An immunohistochemical study of p16, pRb, p21 and p53 proteins in human esophageal cancers. **Anticancer Res**, v.20, p.345-349, 2000.

KAWAKUBO, H. *et al.* Alterations of p53, cyclin D1 and pRB expression in the carcinogenesis of esophageal squamous cell carcinoma. **Oncol Rep**, v.14, n. 6, p. 1453-1459, 2005.

KEMPSTER, S. *et al.* Methylation of exon 2 of p16 is associated with late stage oesophageal cancer. **Cancer Lett**, v.150, p.57-62, 2000.

KERR, J.F.R.; WYLLIE, A.H.; CURRIE, A.R. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. **Br. J. Cancer**, n. 26, p. 239 – 257, 1972.

KERR, J.F.R.; WINTERFORD, C.M.; HARMON, B.V. Apoptosis: its significance in cancer and cancer therapy. **Cancer**, v. 73, p. 2013 - 2026, 1994.

KHUSHALANI, N. Cancer of the esophagus and stomach. **Mayo Clin Proc**, v. 83, n. 6, p.712-722, 2008.

KIM, S.G. *et al.* The expression of p53, p16, cyclin D1 in esophageal squamous cell carcinoma and esophageal dysplasia. **Korean J Gastroenterol**, v. 48, n. 4, p. 269-276, 2006.

KITAMURA, A. *et al.* Reduced Fhit expression occurs in the early stage of esophageal tumorigenesis: no correlation with p53 expression and apoptosis. **Oncology**, v.61, p.205-211, 2001.

KNUDSON, A.G. Hereditary Cancer, oncogenes, and anti-oncogenes. **Cancer Res**, n45, p. 1437 – 1443, 1985.

KOBAYASHI, M. *et al.* p53 Mutation analysis of low-grade dysplasia and high-grade dysplasia/carcinoma in situ of the esophagus using laser capture microdissection. **Oncology**, v. 71, n. 3-4, p. 237-245, 2006.

KOCH, W.M. *et al.* TP53 gene mutations as markers of tumor spread in synchronous oral cancer. **Arch Otolaryngol. Head Neck Surg**, n. 120, p. 943 – 947, 1994.

KOUNTOURAS, J.; ZAVOS, C.; CHATZOPOULOS, D. Apoptosis and autoimmunity as proposed pathogenetic links between *Helicobacter pylori* infection and idiopathic achalasia. **Medical Hypotheses**, v.63, p.624-629, 2004.

KRAICHELY, R.E.; FARRUGIA, G. Achalasia: physiology and etiopathogenesis. **Dis Esophagus**, v.19, p. 213-223, 2006.

KRAJEWSKA, M. *et al* (1997) Immunohistochemical analysis of in vivo patterns of expression of CPP32 (Caspase-3), a cell death protease. **Cancer Res**, 57(8):1605-1613.

KROEMER, G. Mitochondrial control of apoptosis: an introduction. **Biochemical and Biophysical Research Communications**, n. 304, p. 433 - 435, 2003.

KRÜGER, S. *et al.* P16 Immunoreactivity Is an Independent Predictor of Tumor Progression in Minimally Invasive Urothelial Bladder Carcinoma. **European Urology**, v.47 p.463–467, 2005.

KUERBITZ, S.J. *et al.* Wild type p53 is a cell cycle checkpoint determinant following irradiation. **Proc Natl Acad Sci USA**, v.89, p. 7491-7495, 1992.

KUROKI, T. *et al.* Allele loss and promoter hypermethylation of *VLH*, *RAR-beta*, *RASSF1A*, and *FHIT* tumor suppressor genes on chromosome 3p in esophageal squamous cell carcinoma. **Cancer Res**, v. 63, p. 3724-8, 2003.

KWONG, D. *et al.* Chromosomal aberrations in esophageal squamous cell carcinoma among Chinese: gain of 12p predicts poor prognosis after surgery. **Hum Pathol**, v. 35, n. 3, p. 309-316, 2004.

LAM, AK. Molecular biology of esophageal squamous cell carcinoma. **Critical Reviews in Oncology:Hematology**, v.33, p.71–90, 2000.

LANA, M. de; TAFURI, W.L. Doença de Chagas. *In*: NEVES, D.P. **Parasitologia Humana**. 10^a ed. São Paulo: Atheneu, 2000. cap.11, p.428.

LANE, D. P. p53: guardian of the genome. **Nature**, n. 358, p.15 – 16, 1992.

LANE, D. P. A death in the life of p53. **Nature**, n. 362, p. 786 – 787, 1994.

LEBE, B. *et al.* The importance of cyclin D1 and Ki67 expression on the biological behavior of pancreatic adenocarcinomas. **Pathology – research and Prattice**, v. 200, p.389-396, 2004.

LEHMAN, M.B. *et al.* Squamous mucosal alterations in esophagectomy specimens from patients with end-stage achalasia. **Am J Surg Pathol**, v.25, p.1413-1418, 2001.

LEIBY, D.A. *et al.* Seroepidemiology of *Trypanosoma cruzi*, etiologic agent of Chagas disease, in US blood donors. **J Infect Dis**, v.176, p.1047-1052, 1997.

LEVINE, A.J. *et al.* The 1993 Walter Hubert Lecture: The role of p53 tumor-suppressor gene in tumorigenesis. **Br J Cancer**, v.69, p. 409 – 416, 1994.

LEVINE, A.J. p53, the cellular gatekeeper for growth and division. **Cell**, n. 88, p. 323 – 331, 1997.

LI, L.; YANG, T. Expression of Cdk4, cyclin D1 and Rb in exogenous wild type P16 gene transfected bladder cancer cell line. **Colloids and Surfaces B: Biointerfaces**, v.30, p.315–322, 2003.

LIN, D.C.; DU, X.L.; WANG, M.R. Protein alterations in ESCC and clinical implications: a review. **Dis Esophagus**, *in press*, 2008.

MADER, A.M.A.A. *et al.* Esofagite de refluxo em crianças: estudo histológico e morfométrico. **Arq Gastroenterol**, v.39, n. 2, p. 126-131, 2002.

MAESAWA, C. *et al.* Inactivation of the CDKN2 gene by homozygous deletion and de novo methylation is associated with advanced stage esophageal squamous cell carcinoma. **Cancer Res**, v.56, p.3875-3878, 1996.

MALTZMAN, W.; CZYZYK, L. UV irradiation stimulates levels of p53 cellular tumor antigen in nontransformed mouse cell. **Mol Cell Biol**, v.4, p.1689-1694, 1984.

MANDARD, A. M. *et al.* Expression of p53 in oesophageal squamous epithelium from surgical specimens resected for squamous cell carcinoma of the oesophagus, with special reference to uninvolved mucosa. **J Pathol**, v. 181, p. 334-5, 1998.

MANDARD, A.M.; HAINAUT, P.; HOLLSTEIN, M. Genetic steps in the development of squamous cell carcinoma of the esophagus. **Mutat Res**, v. 462, p. 335-342, 2000.

MANOEL-CAETANO, F.S. *et al.* Cytogenetic alterations in chagasic achalasia compared to esophageal carcinoma. **Cancer Genet Cytogenet**, v.149, p. 17 – 22, 2004.

MANOEL-CAETANO, F.S.; SILVEIRA A.F.P.; SILVA, A.E. (2009) Gene mutations in esophageal mucosa of Chagas disease patients. **Anticancer Res**, *in press*.

MASTRACCI, L. *et al.* Cell proliferation of squamous epithelium in gastro-oesophageal reflux disease: correlations with clinical, endoscopic and morphological data. **Aliment Pharmacol Ther**, v. 25, p. 637–645, 2006.

MATSUO, K. *et al.* Gene-environment interaction between an aldehyde dehydrogenase-2 (ALDH2) polymorphism and alcohol consumption for the risk of esophageal cancer. **Carcinogenesis**, v.22, p.913-916, 2001.

MAYAMA, T. *et al.* Frequent loss of copy number on the long arm of chromosome 21 in human esophageal squamous cell carcinoma. **Int J Oncol**, v.17, n.2, p.245-252, 2000.

McCABE, M.L.; DLAMINI, Z. The molecular mechanisms of oesophageal cancer. **Int Immunopharmacol**, v.5, p.1113– 1130, 2005.

MENIN, C. *et al.* Anomalous transcripts and allelic deletions of the FHIT gene in human esophageal cancer. **Cancer Genet Cytogenet**, v.119, p.56-61, 2000.

MICHAEL, D. *et al.* Frequent deletions of FHIT and FRA3B in Barrett's metaplasia and esophageal adenocarcinomas. **Oncogene**, v.15, p.1653-1659, 1997.

MIMURA, K. *et al.* Frequencies of HER-2/neu expression and gene amplification in patients with oesophageal squamous cell carcinoma. **Br J Cancer**, v. 92, n. 7, p. 1253-60, 2005.

MINCIS, M. **Gastroenterologia e hepatologia, diagnóstico e tratamento**. São Paulo: Lemos Editorial & Gráficos Ltda, 1997, 960p. ISBN: 13: 9788561125066

MORI, T. *et al.* Frequent somatic mutation of the MTS1/CDK4I (multiple tumor suppressor/cyclin-dependent kinase 4 inhibitor) gene in esophageal squamous cell carcinoma. **Cancer Res**, v.54, p.3396-3397, 1994.

MORI, M. *et al.* Altered expression of Fhit in carcinoma and precarcinomatous lesions of the esophagus. **Cancer Res**, v.60, p.1177-1182, 2000.

MORITA, M. *et al.* Expression of FHIT in esophageal epithelium and carcinoma: reference to drinking, smoking and multicentric carcinogenesis. **Anticancer Res**, v. 26, n.3B, p.2243-2248, 2006.

MORRIS, R.G. *et al.* Hormone-induced cell death: surface changes in thymocytes undergoing apoptosis. **Am J Pathol**, n. 115, p. 426 – 436, 1984.

MUÑOZ, N. Le dépistage du cancer de l'oesophage est-il réalisable? **Semaines des Hôpitaux de Paris**, v. 73, p. 633-635, 1997.

MURPHY, S.J. *et al.* Have patients with esophagitis got an increased risk of adenocarcinoma? Results from a population-based study. **World J Gastroenterol**, v. 11, p. 7290-7295, 2005.

MURTAZA, I. *et al.* A study on p53 gene alterations in esophageal squamous cell carcinoma and their correlation to common dietary risk factors among population of the Kashmir valley. **World J Gastroenterol**, v.12, n.25, p.4033-4037, 2006.

MUZEAU, F. *et al.* Loss of heterozygosity on chromosome 9 and p16 (MTS1, CDKN2) gene mutations in esophageal cancer. **Int J Cancer**, v.72, p.27-30, 1997.

NAKAKUKI, K. *et al.* Novel targets for the 18p11.3 amplification frequently observed in esophageal squamous cell carcinoma. **Carcinogenesis**, v. 23, n. 1, p. 19-24, 2002.

NOGUCHI, T. *et al.* FHIT expression and hypermetilation in esophageal squamous cell carcinoma. **Int J of Mo Medicine**, v.11, p.441-447, 2003.

NUOVO, G. *et al.* *In situ* detection of the hypermethylation-induced inactivation of the *p16* gene as an early event in oncogenesis. **PNAS**, v.96, n.22, p.12754-12759, 1999.

OHTA, M. *et al.* The FHIT gene, spanning the chromosome 3p14.2 fragile site and renal carcinoma-associated t(3;8) breakpoint, is abnormal in digestive tract cancers. **Cell**, v.84, p.587-597, 1996.

OLIVEIRA, R.B. de *et al.* Gastrointestinal manifestations of Chagas' Disease. **Am J Gastroenterol**, v.93, p.884-889, 1998.

OLIVIER, M. *et al.* The IARC TP53 database: new online mutation analysis and recommendations to users. **Hum Mutat**, v. 19, p. 607-14, 2002.

ORALCHELATION, www.oralchelation.com/faq/images2/a1.gif, Acesso em 10 de março de 2009.

PACK, S.D. *et al.* Molecular cytogenetic fingerprinting of esophageal squamous cell carcinoma by comparative genomic hybridization reveals a consistent pattern of chromosomal alterations. **Genes Chromosomes Cancer**, v.25, n.2, p.160-168, 1999.

PAJECKI, D. *et al.* Megaesophagus Microbiota: A Qualitative and Quantitative Analysis. **J Gastrointest Surg**, v. 6, p. 723 – 729, 2002.

PALLARDY, M.; PERRIN-WOLFF, M.; BIOLA, A. Cellular Stress and Apoptosis. **Toxicol in Vitro**, 11: 573 - 578, 1997.

PAROLIN, M.B.; REASON I.J.M. Apoptose como mecanismo de lesão nas doenças hepatobiliares. **Arq Gastroenterol**, v.38, n.2, p.138-144, 2001.

PEKARSKY, Y. *et al.* *FHIT*: from gene discovery to cancer treatment and prevention. **Lancet Oncol**, v.3, p.748-754, 2002.

PELUCCHI, C. *et al.* Alcohol and tobacco use, and cancer risk for upper aerodigestive tract and liver. **Eur J Cancer Prev**, v.17, n. 4, p.340-344, 2008.

PIMKHAOKHAM, A. *et al.* Nonrandom Chromosomal Imbalances in Esophageal Squamous Cell Carcinoma Cell Lines: Possible Involvement of the *ATF3* and *CENPF* Genes in the 1q32. **Amplico Jpn J Cancer Res**, v. 91, p.1126–1133, 2000.

PINOTTI, H.W. Megaesôfago Chagásico. *In*: COELHO, J.C.U. **Aparelho Digestivo**. 2^a ed. Rio de Janeiro: MDESI, 1996, cap. 1, p.61-67.

PUTZ, A. *et al.* TP53 mutation pattern of esophageal squamous cell carcinomas in a high risk area. **Int J Cancer**, v.98, p.99-105, 2002.

QIN, Y.R. *et al.* Comparative genomic hybridization of esophageal squamous cell carcinoma and gastric cardia adenocarcinoma in high-incidence region of esophageal carcinoma. **Linzhou Henan. Zhonghua Yi Xue Yi Chuan Xue Za Zhi**, v. 21, n. 6, p. 625-8, 2004.

QIN, Y.R. *et al.* Genomic changes in primary lesion and lymph node metastases of esophageal squamous cell carcinoma. **Ai Zheng**, v. 24, n. 9, p.1048-1053, 2005a.

Qin Y.R. *et al.* Comparative genomic hybridization: the profile of chromosomal imbalances in esophageal squamous cell carcinoma. **Zhonghua Bing Li Xue Za Zhi**, v. 34, n. 2, p. 80-83, 2005b.

QIN, Y.R. *et al.* Comparative genomic hybridization analysis of genetic aberrations associated with development of esophageal squamous cell

carcinoma in Henan, China. **World J Gastroenterol**, v. 14, n. 12, p.1828-1835, 2008.

RALHAN, R. *et al.* Frequent alterations in the expression of tumor suppressor genes p16INK4A and pRb in esophageal squamous cell carcinoma in the Indian population. **J Cancer Res Clin Oncol**, v.126, p.655-660, 2000.

RIBEIRO, Jr.U. *et al.* Risk factors for squamous cell carcinoma of the oesophagus. **Br J Surg**, v. 83, p. 1174-1185, 1996.

ROTH, M.J. *et al.* Genetic progression and heterogeneity associated with the development of esophageal squamous cell carcinoma. **Cancer Res**, v.61, p.4098-4104, 2001.

ROTH, M.J. *et al.* p16, MGMT, RARbeta2, CLDN3, CRBP and MT1G gene methylation in esophageal squamous cell carcinoma and its precursor lesions. **Oncol Rep**, v.15, n.6, p.1591-1597, 2006.

ROZ, L. *et al.* Restoration of fragile histidine triad (*FHIT*) expression induces apoptosis and suppresses tumorigenicity in lung and cervical cell lines. **PNAS**, v.99, n. 6, p. 3615 – 3620, 2002.

SANTIN, A. D. *et al.* Gene expression profiles of primary HPV16- and HPV18-infected early stage cervical cancers and normal cervical epithelium: identification of novel candidate molecular markers for cervical cancer diagnosis and therapy. **Virology**, v.331, p.269–291, 2005.

SARD, L. *et al.* The tumor-suppressor gene *FHIT* is involved in the regulation of apoptosis and in cell cycle control. **Proc Natl Acad Sci USA**, v. 96, p. 8489 – 8492, 1999.

SEPEHR, A. *et al.* Distinct pattern of *TP53* mutations in squamous cell carcinoma of the esophagus in Iran. **Oncogene**, v. 21, p. 7368-74, 2002.

SHAW, P *et al.* Induction of apoptosis by wild type p53 in a human colon tumor-derived cell line. **Proc Natl Acad Sci USA**, v.89, p.4495-4499, 1992.

SHI, S. *et al.* Role of p53 gene mutations in human esophageal carcinogenesis: results from immunohistochemical and mutation analyses of carcinomas and nearby non-cancerous lesions. **Carcinogenesis**, v.20, p.591-597, 1999.

SHIMADA, Y. *et al.* Characterization of 21 newly established esophageal cancer cell lines. **Cancer**, v.69, p.277-284, 1992.

SHIMADA Y.; IMAMURA M. Prognostic factors for esophageal cancer--from the viewpoint of molecular biology. **Gan To Kagaku Ryoho**, v. 23, n. 8, p. 972-981, 1996.

SHIMADA, Y. *et al.* Loss of fragile histidine triad gene expression is associated with progression of esophageal squamous cell carcinoma, but not with the patient's prognosis and smoking history. **Cancer**, v.89, p.5 -11, 2000.

SHIOZAKI, H. *et al.* Larynx preservation in surgical treatment of cervical esophageal cancer--combined procedure of laryngeal suspension and cricopharyngeal myotomy. **Dis Esophagus**, v. 13, n. 3, p. 213-218, 2000.

SHINOMIYA, T. *et al.* Comparative genomic hybridization of squamous cell carcinoma of the esophagus: The possible involvement of the *DP1* gene in the 13q34 amplicon. **Genes Chromosomes Câncer**, v.24, p.337-344, 1999.

SILVEIRA, A.P.F. **Investigação de mutações nos genes *TP53* e *FHIT* em esofagite crônica e carcinoma esofágico**. 2008. Dissertação (Mestrado em Genética) – Instituto de Biociências, Letras e Ciências Exatas, Universidade Estadual Paulista “Júlio de Mesquita Filho”, São José do Rio Preto.

SOMA, T. *et al.* Nicotine induces the fragile histidine triad methylation in human esophageal squamous epithelial cells. **Int J Cancer**, v.119, p.1023-1027, 2006.

SOMERS, K.D.; SCHECHTER, G.L. Genetic alterations in head and neck cancer. **Mol Biol Genetics**, v. 5, n. 25, p. 1065 – 1071, 1992.

SOUTO, J.V.Jr.; RIBEIRO, M.A.A. Saúde e vida *on line*/Doença de Chagas. Disponível em: <http://www.nib.unicamp.br/svol/chagas.htm>. Acesso em 26 de outubro de 2005.

SPEICHER, M.R.; CARTER, N.P. The new cytogenetics: blurring the boundaries with molecular biology. **Nature**, v. 6, p. 782 – 792, 2005.

STEELE, R. J. *et al.* The p53 tumour suppressor gene. **Br J Surg**, v. 11, n. 85, p. 1460 – 1467, 1998.

STEMMERMANN, G. *et al.* The molecular biology of esophageal and gastric cancer and their precursors: oncogenes, tumor suppressor genes, and growth factor. **Hum Pathol**, v.25, p.968 – 981, 1994.

SU, M. *et al.* Comparative genomic hybridization of esophageal adenocarcinoma and squamous cell carcinoma cell lines. **Dis Esophagus**, v. 19, n. 1, p.10-14, 2006.

SUGIMOTO, T. *et al.* Integrated analysis of expression and genome alteration reveals putative amplified target genes in esophageal cancer. **Oncol Rep**, v. 18, p. 465-472, 2007.

SUGIURA, K. *et al.* Co-expression of aFGF and FGFR-1 is predictive of a poor prognosis in patients with esophageal squamous cell carcinoma. **Oncol Rep**. v. 17, n.3, p.557-564, 2007.

SUNPAWERA VONG, P. *et al.* Epidermal growth factor receptor and cyclin D1 are independently amplified and overexpressed in esophageal squamous cell carcinoma. **J Cancer Res Clin Oncol**, v.131, n.2, p.111-119, 2005.

TAKAHASHI, K. *et al.* Serum p53 antibody as a predictor of early recurrence in patients with postoperative esophageal squamous cell carcinoma. **Dis Esophagus** v. 20, p. 117–122, 2007.

TAKEUCHI, H. *et al.* Further evidence that altered p16/CDKN2 gene expression is associated with lymph node metastasis in squamous cell carcinoma of the esophagus. **Oncol Rep**, v.8, p.627-632, 2001.

TAKEUCHI, H. *et al.* Cell-cycle regulators and the Ki-67 labeling index can predict the response to chemoradiotherapy and the survival of patients with locally advanced squamous cell carcinoma of the esophagus. **Ann Surg Oncol**, v. 10, n. 7, 2003.

TANAKA, H. *et al.* Methylation of the 5' CpG island of the FHIT gene is closely associated with transcriptional inactivation in esophageal squamous cell carcinomas. **Cancer Res**, v.58, p.3429-3434, 1998.

TRAPASSO, F. *et al.* Designed *FHIT* alleles establish that Fhit-induced apoptosis in cancer cells is limited by substrate binding. **PNAS**, v.100, n. 4 p. 1592 – 1597, 2003.

UEDA, N.; SHAH, S. Apoptosis. **J Lab Clin Med**, 124: 169 - 177, 1994.

UFRGS, Universidade Federal do Rio Grande do Sul. *Trypanosoma cruzi*. Disponível em:
http://www.ufgrs.br/parasite/Imagenatlas/Protozoa/Trypanosoma_cruzi.htm
 Acesso em 11 de dezembro de 2008.

WANG, G.Q. *et al.* Histological precursors of oesophageal squamous cell carcinoma: results from a 13 year perspective follow up study in a high risk population. **Gut**, v. 54, p. 187-192, 2005.

WEINBERG, R.A. **A biologia do câncer**. Porto Alegre: Artmed, 2008. 864p. ISBN:978-85-363-1348-1

WHO, World Health Organization. Chagas. Disponível em:
<http://who.int/ctd/chagas/disease.htm>. Acesso em 11 de dezembro de 2008.

WYLLIE, A.H. Apoptosis. **Br J Cancer**, v. 67, p. 205 – 208, 1993.

XING, E.P. *et al.* Mechanisms of inactivation of $p14^{ARF}$, $P15^{INK4b}$, and $p16^{INK4a}$ genes in human esophageal squamous cell carcinoma. **Clin Cancer Res**, v.5, p.2704-2713, 1999.

XU, F.P. *et al.* *SRC-3/AIB1* protein and gene amplification levels in human esophageal squamous cell carcinomas. **Cancer Lett**, v. 245, n. 1-2, p. 69-74, 2007.

XU, F.P. *et al.* Role of AIB1 gene in development of esophageal squamous cell carcinoma and its clinicopathologic significance. **Zhonghua Bing Li Xue Za Zhi**, v. 37, n. 4, p. 250-254, 2008.

XUE, L.Y. *et al.* Tissue microarray analysis reveals a tight correlation between protein expression pattern and progression of esophageal squamous cell carcinoma. **BMC Cancer**, v. 6, p. 296-311, 2006.

YANG, Y.L. *et al.* Amplification of PRKCI, located in 3q26, is associated with lymph node metastasis in esophageal squamous cell carcinoma. **Genes Chromosomes Cancer**. v. 47, n. 2, p.127-136, 2008.

YEN, C.C. *et al.* Comparative genomic hybridization of esophageal squamous cell carcinoma: correlations between chromosomal aberrations and disease progression/prognosis. **Cancer**, v. 92, n. 11, p.2769-2777, 2001.

YEN, C.C. *et al.* Genotypic analysis of esophageal squamous cell carcinoma by molecular cytogenetics and real-time quantitative polymerase chain reaction. **Int J Oncol**, v. 23, p. 871-881, 2003.

YEN, C.C. *et al.* Copy number changes of target genes in chromosome 3q25.3-qter of esophageal squamous cell carcinoma: TP63 is amplified in early carcinogenesis but down-regulated as disease progressed. **World J Gastroenterol**. v. 11, n. 9, p.1267-1272, 2005.

YONISH-ROUACH E. et al. Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6. **Nature**, v.352, p.345-347, 1991.

ZHANG, G.H. *et al.* Analysis of basement membrane structure and inflammation during the development of esophageal squamous cell carcinoma in the Chinese Chaoshan high risk region. **Cancer Invest**, v. 26, n. 3, p.296-305, 2008.

ZHOU, X. *et al.* Genomic DNA and messenger RNA expression alterations of the *CDKN2B* and *CDKN2A* genes in esophageal squamous carcinoma cell lines. **Genes Chromosomes Cancer**, v.13, p.285-290, 1995.

ZOU, T.T. *et al.* FHIT gene alterations in esophageal cancer and ulcerative colitis (UC). **Oncogene**, v.15, p.101-105, 1997.

Apêndices

Apêndice 1: Termo de Consentimento Livre e Esclarecido (Sanduíche)

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO
(Conselho Nacional de Saúde, Resolução 196/96)

Eu, Marilanda Ferreira Bellini, RG: 30.212.578-4 SSP/SP, aluna de Doutorado do Programa de Pós-Graduação em Genética do Instituto de Biociências, Letras e Ciências Exatas – IBILCE / UNESP – Campus São José do Rio Preto, convido _____

RG: _____, nascido em ____/____/____ e domiciliado à _____, município de _____, usuário (ou responsável legal pelo usuário _____), a participar, como voluntário, no projeto de pesquisa

“Investigação de perdas e ganhos genômicos em pacientes com Megaesôfago Chagásico, pela técnica FISH (Hibridização *in situ* fluorescente) e desenvolvimento de sondas de DNA como marcadores de tumorigênese”, e em extensões deste, que pretende estudar alterações na expressão do material genético, pelas técnicas CGH e FISH em pessoas que apresentam um tipo de doença que provoca o aumento do esôfago, causado pela doença de Chagas, por isso sendo conhecida como “megaesôfago chagásico”. Declaro que o voluntário foi satisfatoriamente esclarecido que: A) o estudo será realizado utilizando pequenos pedaços (biópsias) de local do esôfago com alteração (megaesôfago chagásico), que serão colhidos durante o processo de rotina para a realização de exame de endoscopia, solicitado pelo médico do voluntário, no Serviço de Endoscopia do Hospital de Base de São José do Rio Preto; B) não haverá nenhum risco adicional para sua saúde, pois a colheita do material será realizada pelos profissionais qualificados e que precisa fazer a endoscopia independente deste projeto; C) o voluntário poderá consultar as pesquisadoras responsáveis em qualquer época para esclarecimento de qualquer dúvida; D) está livre para, a qualquer momento, deixar de participar da pesquisa e que não precisa apresentar justificativas para isso; E) fornecerá informações através de um questionário realizado durante uma entrevista antes do seu exame e também autoriza a coleta de dados em seu prontuário médico; F) todas as informações fornecidas pelo voluntário e os resultados obtidos serão mantidos em sigilo e que estes últimos serão utilizados somente para divulgação em reuniões e revistas científicas, sem sua identificação, pois o material coletado será identificado por código; G) será informado pelo seu médico dos resultados obtidos, caso possam auxiliar seu tratamento ou prevenir a progressão da lesão que apresenta no esôfago, independentemente do fato destes poderem mudar seu consentimento em participar da pesquisa; H) que a biópsia só será utilizada após o procedimento cotidiano de emblocamento em parafina pelo Setor de Patologia do Hospital de Base de São José do Rio Preto, responsável pelo seu armazenamento; I) este estudo consiste numa pesquisa científica importante, pois seus resultados poderão trazer informações básicas, evidenciando a expressão diferencial de proteínas, envolvidas nos estágios iniciais que ocorrem nestes tipos de lesões do esôfago (esofagite crônica e megaesôfago chagásico), e assim, se há um risco para o aparecimento de um câncer do esôfago, portanto podendo auxiliar futuramente no diagnóstico precoce, evolução e tratamento da doença.

Assim, o usuário consente em participar do projeto de pesquisa em questão.

São José do Rio Preto, ____ de ____ de 200 ____

Usuário/ responsável legal

Pesquisador responsável

OBS: Este termo apresenta duas vias, uma destinada ao usuário ou seu representante legal e a outra ao pesquisador.

Projeto: Investigação de perdas e ganhos genômicos em pacientes com Megaesôfago Chagásico, pela técnica FISH (Hibridização *in situ* fluorescente) e desenvolvimento de sondas de DNA como marcadores de tumorigênese

Pesquisadores: Doutoranda Marilanda Ferreira Bellini

Profa. Dra. Ana Elizabete Silva

Instituição: UNESP – Universidade Estadual Paulista/IBILCE - Instituto de Biociências, Letras e Ciências Exatas

Endereço: R.Cristóvão Colombo, 2265 - Jardim Nazareth – CEP 15054-000 São José do Rio Preto-SP

Fone: 17 3221-2384

E-mail: mbellini@webmail.ibilce.unesp.br

Apêndice 2: Questionário

Questionário do Projeto: “Investigação de perdas e ganhos genômicos em pacientes com megaesôfago chagásico, pela técnica de FISH (Hibridização *in situ* fluorescente) e desenvolvimento de sondas de DNA como marcadores de tumorigênese”

Responsáveis: M.sc. Marilanda Ferreira Bellini, Profa. Dra. Ana Elizabete Silva (Departamento de Biologia – IBILCE/UNESP – São José do Rio Preto – SP) e Dr. Kenji Miyazaki (Setor de Endoscopia – Hospital de Base, São José do Rio Preto)

IDENTIFICAÇÃO:

Nome: _____ Prontuário: _____
 Data de nascimento: ____/____/____ Sexo: () M () F
 Endereço Atual: _____ Fone: _____
 Cidade: _____ Estado: _____
 Procedência: _____
 Profissão Atual: _____ Tempo de atuação: _____
 Profissão Anterior: _____ Tempo de atuação: _____

DADOS PESSOAIS E FAMILIAIS

Consumo de bebida alcoólica: () Sim () Não
 Há quantos anos? _____ Tipo de bebida: _____ dose: _____

Consumo de cigarro: () Sim () Não
 Há quantos anos? _____ Quantidade (unidade/dia): _____

Doenças Anteriores no Aparelho Digestório:

() esôfago () estômago Outras: _____ Tipo: _____

Tratamentos anteriores para megaesôfago: () Sim () Não Tipo: _____

Cirurgias anteriores: () Sim () Não Tipo: _____

Há história de tumor de esôfago ou outras doenças na família: () Sim () Não

Tipo: _____ Grau de Parentesco: _____

Data: ____/____/____ Responsável pelo Procedimento: _____

Apêndice 3: Caracterização das amostras de mucosa esofágica normal e Carcinoma de células escamosas de esôfago

Caracterização de estilo de vida e clínico-patológica nos grupos de pacientes com mucosa esofágica normal (NM) e com carcinoma de células escamosas de esôfago (ESCC), acompanhados por 4 anos.

Código	Idade (anos)	Sexo	Etilismo	Tabagismo	Dados Clínicos
Mucosa Esofágica Normal					
NM1	26	F	Não	Não	Nada Consta
NM2	67	F	Não	Não	Nada Consta
NM3	33	M	Sim	Sim	Nada Consta
NM4	29	F	Não	Não	Nada Consta
NM5	51	M	Sim	Sim	Nada Consta
NM6	34	M	Sim	Não	Nada Consta
NM7	34	F	Sim	Não	Nada Consta
NM8	66	F	Não	Não	Nada Consta
NM9	54	F	Não	Não	Nada Consta
NM10	30	F	Não	Sim	Nada Consta
Carcinoma de células escamosas de esôfago					
ESCC1	65	M	Sim	Sim	Cardiopatia, megacólon, megaesôfago
ESCC2	56	M	Sim	Sim	Sorologia positiva (Chagas)
ESCC3	69	F	Sim	Não	Megacólon, megaesôfago, cardiopatia
ESCC4	42	M	Não	Sim	Tumor intestinal operado
ESCC5	48	M	Sim	Sim	Nada Consta
ESCC6	64	M	Sim	Sim	Nada Consta
ESCC7	47	M	Sim	Sim	Nada Consta
ESCC8	55	M	Sim	Sim	Nada Consta
ESCC9	64	M	Não	Sim	Nada Consta
ESCC10	48	M	Sim	Sim	Nada Consta
ESCC11	63	M	Não	Não	gastrite enantematosa

F, Feminino; M, Masculino

Apêndice 4: Caracterização das amostras de pacientes com esofagite crônica

Caracterização de estilo de vida e clínico-patológica nos grupos de pacientes com esofagite crônica (CE), acompanhados por 4 anos.

Código	Idade (anos)	Sexo	Etilismo	Tabagismo	Dados Clínicos
CE1	40	F	Não	Não	Nada Consta
CE2	43	F	Não	Sim	Nada Consta
CE3	50	F	Não	Sim	Cardiopatias, obesidade, óbito
CE4	52	M	Sim	Sim	Hérnia Hiato, gastrite leve
CE5	40	M	Sim	Não	Malária
CE6	42	F	Não	Não	Nada Consta
CE7	41	F	Não	Não	Nada Consta
CE8	49	F	Não	Não	Nada Consta
CE9	57	F	Não	Não	Nada Consta
CE10	56	M	Não	Não	Nada Consta
CE11	65	M	Não	Não	Gastrite enantematosa
CE12	51	M	Não	Não	Gastrite enantematosa, <i>H. pylori</i> +
CE13	55	M	Não	Não	Nada Consta
CE14	67	M	Não	Não	Cardiopatia
CE15	54	M	Não	Não	Gastrite enantematosa
CE16	46	F	Não	Não	Gastrite discreta
CE17	40	F	Não	Não	Nada Consta
CE18	47	M	Não	Não	Gastrite leve; úlcera duodenal recorrente
CE19	42	M	Não	Não	Nada Consta
CE20	70	F	Não	Não	Nada Consta
CE21	45	F	Não	Sim	Carcinoma de cólo uterino
CE22	41	F	Não	Não	Cálculo renal
CE23	68	M	Não	Sim	Câncer de próstata

F, Feminino; M, Masculino

Apêndice 5: Caracterização das amostras de pacientes chagásicos sem megaesôfago.

Caracterização de estilo de vida e clínico-patológica no grupo de paciente chagásico sem megaesôfago (CD), acompanhados por 4 anos.

Código	Idade (anos)	Sexo	Etilismo	Tabagismo	Dados Clínicos
CD1	63	M	Sim	Não	Esôfago de Barret; megacólon operado
CD2	62	M	Sim	Não	Megacólon; esofagite crônica leve
CD3	62	F	Não	Não	Cardiopatia; esofagite crônica leve
CD4	72	F	Não	Sim	Esofagite crônica leve
CD5	76	F	Não	Não	Esofagite crônica leve
CD6	49	M	Não	Sim	Esofagite crônica moderada
CD7	45	F	Não	Não	Megacólon; esofagite crônica leve
CD8	75	F	Sim	Sim	Esofagite crônica moderada, gastrite leve e cardiopatia
CD9	59	M	Sim	Sim	Esofagite leve
CD10	79	F	Não	Não	Nada Consta
CD11	50	F	Não	Sim	Nada Consta
CD12	59	F	Não	Não	Megacólon, esofagite crônica moderada
CD13	57	M	Não	Não	Megacólon, esofagite de refluxo, gastrite erosiva

F, Feminino; M, Masculino

Apêndice 6: Caracterização das amostras de pacientes chagásico com megaesôfago

Caracterização de estilo de vida e clínico-patológica no grupo de paciente chagásico com megaesôfago (CM), acompanhados por 2 a 4 anos.

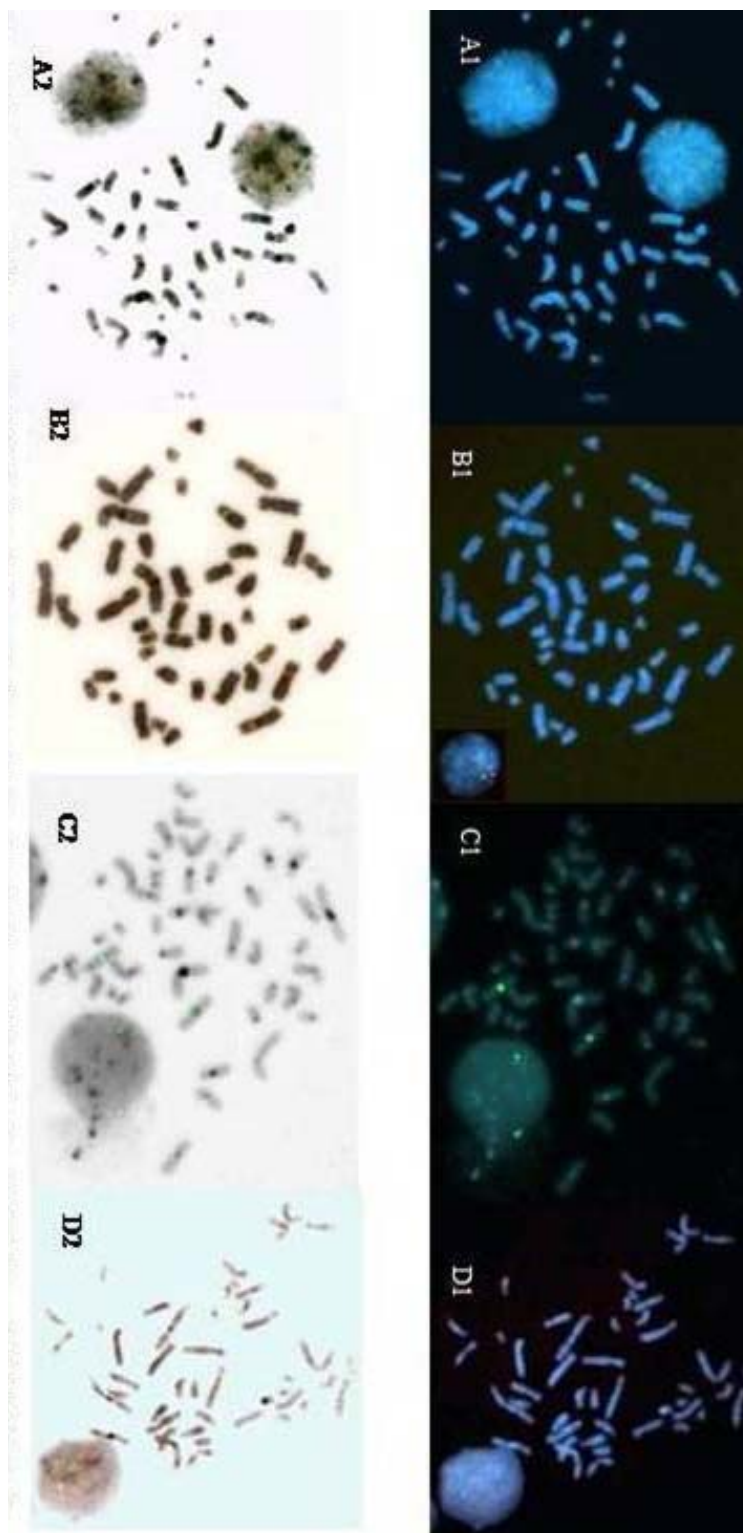
Código Original	Código FISH	Grau CM	Idade (anos)	Sexo	Etilismo	Tabagismo	Dados Clínicos
CM1		II	59	M	Não	Não	Esofagite crônica moderada
CM2		II	58	F	Sim	Não	Megacólon operado, esofagite crônica leve
CM3	CM2	IV	64	F	Não	Sim	Esofagite crônica leve
CM4	CM3	IV	72	M	Não	Não	Megacólon/ esofagite crônica severa
CM5	CM4	I	83	M	Sim	Sim	Megacólon; esofagite crônica severa; óbito
CM6	CM5	III	60	M	Sim	Não	Esofagite crônica leve
CM7	CM6	III	81	F	Sim	Sim	Megacólon operado; cardiopatia; esofagite crônica leve
CM8	CM7	IV	55	F	Não	Sim	Nada Consta
CM9	CM8	IV	61	M	Não	Não	Cardiopatia
CM10	CM9	IV	78	M	Não	Sim	Esofagite crônica severa
CM11	CM10	III	58	F	Não	Não	Gastrite crônica
CM12	CM11	III	63	M	Sim	Sim	Gastrite severa e cardiopatia
CM13	CM12	IV	42	M	Não	Não	Gastrite leve
CM14	CM13	III	69	F	Sim	Sim	Megaesôfago recidivado; cardiopatia; esofagite crônica severa
CM15	CM14	III	70	F	Não	Sim	Esofagite crônica moderada
CM16	CM15	IV	68	F	Não	Não	Esofagite crônica moderada, gastrite crônica severa, cardiopatia
CM17	CM16	IV	83	M	Não	Sim	Esofagite crônica severa
CM18	CM17	III	61	M	Não	Sim	H pylori +, gastrite crônica moderada
CM19	CM18	III	64	M	Sim	Sim	Esofagite crônica moderada, gastrite

							crônica discreta
CM20		III	57	M	Sim	Sim	Gastrite crônica, H pylori+
CM21	CM20	IV	59	F	Não	Sim	Cardiopatia, esofagite crônica moderada
CM22		III	66	F	Não	Não	Esofagite crônica moderada, gastrite enantemática e cardiopatia
CM23	CM21	II	53	F	Não	Sim	Esofagite crônica moderada
	CM22	IV	63	F	Não	Não	Megaesôfago Recidivado
	CM23	I	57	F	Não	Sim	Gastrite, Extração de vesícula biliar, esofagite crônica moderada, cardiopatia
	CM24	III	69	M	S	Sim	Cardiopatia, Hipertensão, Metaplasia Intestinal, Gastrite Crônica Enantematosa
	CM25	III	58	F	Não	Não	Constipação Intestinal, Megacólon, Megabulbo Duodenal, Gastrite Enantematosa
	CM26	III	40	M	Não	Sim	Gastrite Crônica Discreta, H pylori +, Esofagite Crônica Leve, Megacólon e Megaesôfago Recidivados
	CM27	I	59	F	Não	Não	Gastrite enantematosa, duodenite erosiva, metaplasia intestinal
	CM28	II	59	F	Não	Não	Gastrite, Esofagite Erosiva
	CM29	II	64	M	Não	Não	Megaesôfago Recidivado, Megacólon, Esofagite Crônica Moderada, Refluxo Gastro-esofágico
	CM30	III	70	F	Não	Sim	Constipação Intestinal, Megacólon, Gastrectomia Parcial, Colectomia subtotal, gastrite Crônica, Metaplasia Intestinal, Esofagite Crônica Moderada
	CM31	III	40	M	Não	Não	Constipação intestinal, esofagite crônica moderada, gastrite enantematosa

CM32	II	48	F	Não	Não	Constipação Intestinal, Refluxo Gástrico- esofágico, esofagite crônica moderada, pangastrite enamtematosa
CM33	I	64	F	Não	Não	Gastrite Erosiva, Bulboduodenite
CM34	III	53	M	Sim	Não	Esofagite Crônica Moderada, Refluxo Gastro-esofágico
CM35	III	57	M	Sim	Sim	Nada Consta
CM36	II	66	M	Sim	Sim	Constipação Intestinal, Megacólon, Cardiopatia, Gastrite, Hérnia de Hiato
CM37	III		M	Não	Não	Nada Consta
CM39	II	76	F	Não	Não	Cardiopatia
CM19	III	65	M	Sim	Sim	Gastrite Crônica, H pylori+, Metaplasia Intestinal, Cardiopatia
CM40	II	62	M	Não	Sim	Úlcera, Gastrite, Esofagite Crônica Erosiva, Constipação Intestinal
CM1	I	69	F	N	N	Hérnia de Hiato, Gastrite Enantemosa, Úlcera, Esofagite, H pylori +, Cardiopatia
CM38	IV	56	F	Não	Sim	Constipação Intestinal

F, Feminino; M, Masculino

Apêndice 7: Chromosomal Mapping for the newly developed probes.



A1) *FHIT* (red) and *FGFR1* (green);

B1) *TP63* (red) and *PIK3CA* (green);

C1) *pα3.5* (green);

D1) *YES1* (red) and *NCOA3* (green);

Inverted DAPI images.

A2) *FHIT* – 3p14.2 and *FGFR1* – 8p12;

B2) *TP63* – 3q28 and *PIK3CA* – 3q26.32;

C2) *pα3.5* – centromere of chromosome 3;

D2) *YES1* – 18p11.31 and *NCOA3* – 20q12.

Anexos

ANEXOS

Anexo 01. Parecer Consubstanciado do Comitê de Ética em Pesquisa Institucional (Projeto de Doutorado Original).

	UNIVERSIDADE ESTADUAL PAULISTA
“JÚLIO DE MESQUITA FILHO”	
COMITÊ DE ÉTICA EM PESQUISA - CEP PARECER DO PROJETO DE PESQUISA	
IDENTIFICAÇÃO	
Nome do pesquisador: Marilanda Ferreira Bellini Departamento: Biologia Instituição: IBILCE/UNESP/CSJRP Projeto: Avaliação de apoptose, proliferação celular e expressão das proteínas p53, fhit e p16 em lesões esofágicas benignas: esofagite crônica e megaesôfago chagásico	
PARECER CONSUBSTANCIADO	
Dentre as lesões benignas de esôfago destacam-se a esofagite crônica e o megaesôfago chagásico, por se tratarem de lesões promotoras de processos inflamatórios da mucosa, aumentando os riscos de danos ao dna e a carcinogênese. Destaca-se também a influência de fatores ambientais e genéticos associados a esse processo. Considerando as alterações genéticas que podem estar relacionadas com as etapas iniciais da carcinogênese de esôfago justifica-se a necessidade de outros estudos para avaliar desequilíbrios no controle do ciclo celular e fatores patogênicos. Sendo assim os objetivos desse trabalho serão avaliar as alterações do ciclo celular (apoptose e proliferação celular) por meio da expressão da caspase-3 ativada e do antígeno Ki-67; a expressão das proteínas p53, fhit, e p16 em lesões benignas do esôfago, como, esofagite crônica, megaesôfago chagásico, mucosa esofágica de chagásico sem megaesôfago e em carcinoma de esôfago e, sua associação com a infecção pela <i>H. pylori</i>. Serão coletadas amostras de material de arquivo patológico de cerca de 70 indivíduos após ressecção cirúrgica ou biópsia por endoscopia consistindo em 20 amostras de esofagite crônica, 20 amostras de megaesôfago chagásico, 10 amostras de carcinoma de células escamosas do esôfago, 10 amostras de mucosa de chagásico sem megaesôfago e 10 amostras de mucosa esofágica normal obtidas de indivíduos com queixa de dispepsia gástrica submetidos à endoscopia e com análise histopatológica normal. Como o presente projeto é uma extensão de projeto anteriormente aprovado sobre o numero de processo 25.000.067.565-2003-59 sob parecer nº 1538/2003 a pesquisadora solicita dispensa da obtenção de novo termo de consentimento livre e esclarecido, considerando a dificuldade para contato com esses indivíduos, muitos dos quais já foram a óbito. Os termos de consentimento exclusivos para os pacientes com esofagite crônica serão obtidos pela pesquisadora responsável e está apresentando adequadamente. Estão previstas as formas de entrega dos resultados genéticos obtidos através dos médicos dos pacientes e inclusão em seus prontuários. O projeto inclui como metodologias: avaliação imuno-histoquímica, detecção de apoptose e proliferação celular por meio de anticorpo monoclonal; expressão das proteínas por meio de kits específicos e avaliação molecular de <i>H. pylori</i>, por meio de PCR. Serão incluídos no presente projeto indivíduos adultos de ambos os gêneros que concordarem em participar após os devidos esclarecimentos. Como o presente projeto utilizará amostras emblocadas em parafina ou de arquivo e ainda de procedimentos de rotina diagnóstica não acarretará riscos para a saúde dos participantes. Tendo em vista a importância do conhecimento da proliferação celular da manifestação da patogenia e da experiência comprovada da pesquisadora e de sua orientadora somos favoráveis a aprovação do presente projeto nos termos em que foi proposto.	
(X) APROVADO, aguardar apreciação final da CONEP () NÃO APROVADO () SOLICITAR INFORMAÇÕES COMPLEMENTARES	
Datas previstas para apresentação dos relatórios	
1º relatório: 15 /06 / 2006	2º relatório: 15/06 /2007
São José do Rio Preto, 16 de junho de 2005.	
 Prof. Dra. Claudia Regina Bonini Domingos Coordenadora do CEP	

Anexo 02. Parecer Consubstanciado do Comitê de Ética em Pesquisa Institucional (Projeto de Estágio de Doutorado no Exterior).


MINISTÉRIO DA SAÚDE
Conselho Nacional de Saúde
Comissão Nacional de Ética em Pesquisa

OFÍCIO Nº 722 CONEP/ CNS/ MS

Brasília, 25 de abril de 2007.

Senhor (a) Coordenador (a),

Encaminhamos, em anexo, o(s) Parecer (es) nº **357/2007** (já enviado(s) por fax), da Comissão Nacional de Ética em Pesquisa – CONEP, referente(s) a projeto(s) de pesquisa acompanhada(s) por esse Comitê.

Atenciosamente,

P/ Eliane Aparecida Cruz
Secretária Executiva do
Conselho Nacional de Saúde – CONEP

Sr(a). Claudia Regina Bonini Domingos
Coordenador do Comitê de Ética em Pesquisas
Instituto de Biociências Letras e Ciências Exatas/ Campus de São
José do Rio Preto/ IBILCE/UNESP
Rua Cristóvão Colombo 2265
Jardim Nazareth São José do Rio Preto SP
Cep.: 13.054-000

Esplanada dos Ministérios Ministério da Saúde, Bloco "G"- Edifício Anexo, Ala "B" - 1º andar - Sala 145 - 70058-900 -Brasília, DF
Tel.: (61) 33152951 - Telefax: (61) 3226.6453 – E-mail: conep@saude.gov.br – homepage : <http://lconselho.saude.gov.br>

Anexo 03. *Metodologias para o desenvolvimento de sondas utilizadas na técnica de Hibridação In Situ Fluorescente (FISH)*

VI. DNA Cloning - Protocol 1

Establishing cultures, isolating single colonies, and verifying sequences

I. Reagents

1. Agar plates. LB-agar plates must contain the appropriate antibiotic in the correct concentration, based on the library design.

Antibiotics

- a. Ampicillin (Sigma, Cat. # A2804).

Stock solution (1:1000) = 60 mg/ml

Dissolve 1.5 g ampicillin sodium salt in 25 ml of distilled water.

Filter sterilize, dispense in 1ml aliquots in screw cap tubes. Store at -20°C .

Usual final concentration in culture media = 40 ug/ml.

- b. Kanamycin sulfate (Gibco, Cat. # 11815-016).

Stock = 25 mg/ml

Dissolve 250 mg in 10 ml of distilled water.

Filter sterilize, dispense in 1 ml aliquots in screw cap tubes. Store at -20°C .

Usual final concentration in culture media = 25 ug/ml.

- c. Tetracycline hydrochloride (Gibco, Cat. # 11860-012).

Stock = 12.5 mg/ml

Dissolve 125 mg in 10 ml ethanol/water (50% v/v).

Filter sterilize, dispense in 1ml aliquots in screw cap tubes. Store at -20°C in the dark (wrap tubes in aluminum foil).

Usual final concentration in culture media = 12.5 ug/ml.

- d. Penicillin/Streptomycin (SIGMA, Cat. # P0906). Penicillin G potassium = 5,000,000 Units (3.34 g/5millions units = 0.668 g/million units). Streptomycin sulfate = 5 g

Stock = 10,000 U of pen & strep

Dissolve the content of the vial in 500 ml distilled water.

Filter sterilize, dispense in 2 ml aliquots in screw cap tubes. Store at -20°C .

Usual final concentration is 1/10 dilution.

- e. Chloramphenicol (Sigma, Cat # C-0378)

Stock = 34 mg/ml

Dissolve 340 mg in 10 mL of absolute EtOH. Mix solution completely and aliquot into microcentrifuge tubes. Store at -20°C .

2. LB-broth – freshly prepared and supplemented with the appropriate antibiotic (see above for information.).
3. 1% Triton X
4. 20mM Tris HCl 7.5
5. 2mM EDTA
6. BAC clones (recommend <http://www.ensembl.org> OR <http://genome.ucsc.edu> for clone selection)
7. Primers (recommend Integrated DNA Technologies, Coralville, IA 800-328-2661 www.idtdna.com) specific to BACs
8. GoTaq Green Master Mix (Promega, Cat. # M7122)
9. Sterile 50% Glycerol – Combine equal amounts Sterile water and Glycerol, autoclave
10. 1xPBS
11. Cold 100% ethanol and 70% ethanol.



Cytogenetics Core

Anschutz Medical Campus, L18-8401A
Mail Stop 8117 P.O. Box 6511 Aurora, CO 80045
Phones: 303-724-3147, 303-724-3148
Fax: 303-724-3889 www.uccc.info/cytogenetics

207

12. 3 M NaAcetate pH 5.4
13. TE Buffer (10mM Tris-HCl, 0.5mM EDTA, pH 9.0)
14. Ethidium Bromide Stock Solution 10mg/ml
15. Agarose ultraPure (Life Technologies Cat. # 15510-027)
16. 1 x Tris-Acetate Buffer (TAE) – Dilute 1 part 50x stock in 49 parts Milli-Q water

Prepare 50x stock buffer as follows:

242.0 g Tris base
57.1 mL Glacial Acetic Acid
100 mL 0.5M EDTA pH 8.0
Sufficient quantity Milli-Q water for 1L solution]

17. 6x loading dye
18. Markers for size of DNA bands: (a) 100bp High Mass Ladder; (b) 3 ul of Gel Pilot 100 bp ladder (Qiagen # 239035) or (c) 0.5 ul of 100 bp DNA ladder (Invitrogen # 15628-019)

II. Procedure

A. Selecting clones

1. Select the proper clone(s) for the area of interest using
 - a. <http://www.ensembl.org>
 - b. <http://genome.ucsc.edu>
2. Print a hard copy of the Contig View for the Probe Development binder which includes:
 - a. Chromosomal map
 - b. Position of gene of interest
 - c. Collection of clones available
 - d. Highlight selected clone
3. Acquire the clone(s) of interest from one of the following:
 - a. <http://www.CHORI.org>
 - b. <http://www.invitrogen.com>
4. Print a hard copy of the documentation for the clone purchase

B. Establishing cultures from stab or stocks (Following steps should be done using sterile technique.)

1. Prepare LB broth minus antibiotic. Autoclave with Liquid240 setting. Aliquot 3 ml of sterile LB broth into 15ml tube. Incubate the tube overnight at 37°C to test for contamination. If the broth is clear on day two, proceed with the protocol.
2. Determine the amount of LB broth (3 ml per 15ml tube per clone) you will need and supplement this amount with the appropriate antibiotic. Label each tube with clone ID, initials and date.
3. Aliquot 3 ml of antibiotic-supplemented LB broth into 15 ml tubes. Stab the original glycerol stock with a sterile loop, scoop up a few cells and swish loop into the broth. Incubate in shaker waterbath overnight at 37°C.
4. After culturing overnight, freeze 1 ml of culture in two labeled cryovials: 500ul culture + 500ul 50% sterile glycerol and store at -80°C. Indicate location of frozen vials in Freezer -80°C log.
5. Store the remaining amount at 4°C (good for 2 weeks) wrapped or proceed to the next step.

C. Isolating and setting up mini-cultures from single cell colonies

1. Prepare LB agar minus antibiotic. Autoclave with Liquid240 setting. Pour agar plates (100 x 15mm plates hold 35-40ml), allow to solidify and store all but one plate at 4°C. Incubate the



Cytogenetics Core

Anschutz Medical Campus, L18-8401A
Mail Stop 8117 P.O. Box 6511 Aurora, CO 80045
Phones: 303-724-3147, 303-724-3148
Fax: 303-724-3889 www.uccc.info/cytogenetics

208

agar plate overnight at 37°C to test for contamination. If the plate is clear on day two, proceed with protocol.

2. Determine the number of agar plates (2 plates per clone) need, apply 75-100ul of antibiotic on plate surface and spread thoroughly with sterile glass triangle. Allow plates to dry for 2-3 hours. Label each plate with clone, tech initials and date.
3. Stab the original glycerol stock or touch the liquid culture with a sterile loop and streak the surface of the antibiotic LB-agar plate. Streak plates with a small number of turn-arounds as to not facilitate overgrowth.
4. Incubate overnight at 37°C in designated incubator.
5. Transfer 5-10 single bacterial colonies using the end of a transfer pipet tip to 15 ml tubes with 3 ml LB-broth with the appropriate antibiotic – one colony per tube. Label each tube with the clone ID, colony number, initials and date.
6. Store agar plates at 4°C (good for 2 weeks) wrapped in Saran wrap
7. Incubate tubes with loose lids (must allow air flow) in shaker waterbath 8-12h at 37°C.

D. Verifying presence of specific DNA sequences

1. Design primers specific to template
 - a. Search Ensembl (www.ensembl.org) to insure primers are crossing intron/exon junctions; use flanking parameters of 500 (flanking sequence) 25 (intron bp); show full intronic sequence
 - b. Copy and paste selected sequence from Ensembl into Integrated DNA Technologies (IDT) www.idtdna.com to select and purchase forward and reverse primer sequences.
 1. Optimal primers are 400bp, $\leq 50\%$ C/G content, and have annealing temperature ranges from 55-60°C
 2. Blast primers before purchasing directly from IDT webpage.
 - a. Select the following Blast parameters:
Database Human genomic + transcript
Optimize for Highly similar sequences (megablast)
 - b. An optimal Blast exhibits 100% Query Coverage and an E value <1.0 to the area of interest only. If there is 100% Query Coverage and an E value <1.0 to areas different from the chosen, new primer sequences must be selected for optimization.
 3. Once a sequence Blast is successful, print a hard copy of the Blast table (typically 3-8 pages in length) and label the pages “forward” or “reverse”
 4. Print a hard copy of the purchased Primer Set
 - c. Create and print for the Probe Development binder a Word document that includes the following: 1. Name of clone; 2. Name of gene/area; 3. Exon/Intron sequence used for primer design; 4. Forward and Reverse primer - sequences only
2. Prepare PCR templates from single colony cultures lysing the cells as follows:
 - a. Prepare plasmid lysis solution by combining the following for a total volume of 5ml:
 1. 50ul 100% Triton X; 2. 100ul 1M Tris HCl 7.5; 3. 20ul 0.5M EDTA; 4. 4830ul sterile water
 - b. In a microcentrifuge tube add 1. 50 ul plasmid lysis solution; 2. 5 ul of cell suspension
 - c. Heat to 95°C for 5 minutes using the PTC 200 or AB 2720 Thermal Cycler
 - d. Chill on ice or hold at 4°C until ready for PCR
3. Store remaining single colony cultures at 4°C after parafilming the caps shut (good for two weeks).



Cytogenetics Core

Anschutz Medical Campus, L18-8401A
Mail Stop 8117 P.O. Box 6511 Aurora, CO 80045
Phones: 303-724-3147, 303-724-3148
Fax: 303-724-3889 www.uccc.info/cytogenetics

209

4. Submit the lysates to 'Touchdown' PCR (programmed into AB 2720 Thermal Cycler for primers chosen with annealing temperature range from 55-65°C).
 - a. Dilute Primers. For IDT primers, add the vial's nmol value $\mu\text{l} \times 10$ of sterile or nuclease-free water directly into the primer vial.
 - b. Dilute Stock. 10 μl stock primer + 90 μl sterile or nuclease-free water. Be sure to label diluted primer tubes with primer name, forward or reverse, initials and date.
 - c. In a 200 μl microcentrifuge tube add the following reagents in the order listed below:
 - Promega GoTaq Green Master Mix = 12.5 μl
 - Primers = 1 μl each of forward and reverse
 - Sterile Water = 9 μl
 - Template = 1.5 μl
 - Total reaction volume = 25 μl
 - d. Store vials at -20°C or proceed with next step.
5. Verify size of fragment by submitting specimen to gel electrophoresis using a 1% agarose gel to confirm that the product band has the expected size (small gel casting tray 50 ml, larger tray 150 ml + add EtBr prior to pouring the gel - ~4-6 μl of 10mg/ml stock EtBr). Allow gel to cascade for 30-40 mins for a small gel, 45-60 min for a large gel, run at 85-90 volts.
6. In the marker lane, use appropriate size markers (ie 2 μl of 100bp High Mass Ladder, 3 μl of Gel Pilot 100 bp ladder or 0.5 μl of 100 bp DNA ladder Invitrogen +1 μl 6x dye + sterile water to complete 6 μl . In the test DNA wells, use 6 μl of DNA product (dye is part of GoTaq Green Master Mix)
7. Compare the results of the gel for each colony per clone and select for further steps one or two colonies that showed the expected product size and had the sharpest band on the gel.
8. Expand the clone in overnight cultures:
 - a. For mini-cultures: 3 ml LB broth with proper antibiotic + one loopful of culture at 37°C in the water bath shaker.
 - b. For maxi-cultures: Transfer remaining content of 15 ml tube to a 1 liter erlenmeyer and add an additional 125 ml of TB broth and appropriate quantity of antibiotic.
9. Make aliquots of the selected single colony (in duplicate) to freeze: 500 μl single colony culture + 500 μl 50% sterile glycerol and store at -80°C. Indicate location of frozen vials in Freezer - 80°C log.
10. Submit the remaining culture to the appropriate DNA purification protocol.

Revised April 15, 2008 (LG/MS/SH)

Approved on _____ by _____

Read and understood by laboratory personnel

Name

Date

VI. DNA Cloning – Protocol 6

BAC DNA Preparations from Mini-Cultures

I. Reagents

1. QIAamp DNA Mini Kit (Qiagen Cat. # 51304)
2. 1XPBS
3. Cold 100% ethanol and 70% ethanol.
4. 3 M NaAcetate pH 5.4
5. TE Buffer (10mM Tris-HCl, 0.5mM EDTA, pH 9.0)
6. Ethidium Bromide Stock Solution 10mg/ml
7. Agarose ultraPure (Life Technologies Cat. # 15510-027)
8. 1 x Tris-Acetate Buffer (TAE) – Dilute 1 part 50x stock in 49 parts Milli-Q water
Prepare 50x stock buffer as follows: 242.0 g Tris base + 57.1 mL Glacial Acetic Acid +100 mL 0.5M EDTA pH 8.0 + Milli-Q water to complete 1L solution]
9. 6x loading dye
10. Markers for size of DNA bands: (a) 100bp High Mass Ladder; (b) 3 ul of Gel Pilot 100 bp ladder (Qiagen # 239035) or (c) 0.5 ul of 100 bp DNA ladder (Invitrogen # 15628-019)

II. Procedure Note: Following steps should be done using sterile technique

A. Establishing Cultures and Isolating Single Colonies

1. Complete DNA Cloning Protocol 1: Establishing bacterial cultures, isolating single colonies and verifying presence of desired sequence

B. DNA Extraction and Purification

Extract and purify DNA from mini-cultures using the QIAamp DNA mini-kit (Appendix pg.49 and Blood and Body Fluid Spin Protocol pgs.27-29.) The purified DNA is used as template for whole genome amplification using the Qiagen REPLI-g kit. Even though this mini-kit is not designed for BAC DNA, experimentation has resulted in sufficient yields for REPLI-g amplification. For greater BAC DNA yields refer to DNA Protocol 5 using the Nucleobond BAC Maxi-Kit.

1. Centrifuge 2ml of cell suspension for 5 min at 9.0x1000 rpm/g in a microcentrifuge tube. Remove the supernatant completely and discard, taking care not to disturb the cell pellet.
2. Resuspend cell pellet in 1XPBS to a final volume of 200 µl.
3. Add 20 µl QIAGEN Proteinase K.
4. Add 200 µl Buffer AL to the sample. Mix by pulse-vortexing for 15 s. In order to ensure efficient lysis, it is essential that the sample and Buffer AL are mixed thoroughly to yield a homogeneous solution.
5. Incubate in Thermal Block at 56°C for 10 min.
6. Pulse spin the microcentrifuge tube to remove drops from the inside of the lid.
7. Add 200 µl 100% ethanol to the sample, and mix again by pulse-vortexing for 15 s. After mixing, pulse spin the microcentrifuge tube to collect drops from the inside of the lid.
8. Carefully apply the mixture from step 6 to the QIAamp Spin Column (in a 2 ml collection tube) without wetting the rim, close the cap, and centrifuge at 9.0x1000 rpm/g in for 1 min. Place the



Cytogenetics Core

Anschutz Medical Campus, L18-8401A
Mail Stop 8117 P.O. Box 6511 Aurora, CO 80045
Phones: 303-724-3147, 303-724-3148
Fax: 303-724-3889 www.uccc.info/cytogenetics

211

- QIAamp Spin Column in a clean 2ml collection tube (provided), and discard the tube containing the filtrate.
9. Carefully open the QIAamp Spin Column and add 500 μ l Buffer AW1 without wetting the rim. Close the cap and centrifuge at 9.0x1000 rpm/g for 1 min. Place the QIAamp Spin Column in a clean 2 ml collection tube (provided), and discard the collection tube containing the filtrate.
 10. Carefully open the QIAamp Spin Column and add 500 μ l Buffer AW2 without wetting the rim. Close the cap and centrifuge at 9.0x1000 rpm/g for 3 min.
 11. Place the QIAamp Spin Column in a new 2 ml collection tube (not provided) and discard the collection tube with the filtrate. Centrifuge at 9.0x1000 rpm/g for 1 min.
 12. Place the QIAamp Spin Column in a clean 1.5 ml microcentrifuge tube (not provided), and discard the collection tube containing the filtrate. Carefully open the QIAamp Spin Column and add 200 μ l Buffer AE. Incubate at room temperature (15–25°C) for 5 min, and then centrifuge at 9.0x1000 rpm/g for 1min. ***IMPORTANT: After centrifugation, keep the supernatant and label microcentrifuge tube Elute1.
 13. Repeat Step 12 in a second clean 1.5 ml microcentrifuge tube. ***IMPORTANT: After centrifugation, keep the supernatant and label microcentrifuge tube Elute2.

C. DNA Precipitation and Concentration

1. Add content Elute 2 tube to Elute 1 tube for a total volume of 400 μ l.
2. Add to the 400 μ l reaction:
 - a. 0.1x the volume of the reaction of 3M Na Acetate = 40 μ l
 - b. 2-2.5x of the volume of reaction of 100% cold EtOH = ~1100 μ l
3. Mix gently but thoroughly by inverting the tube and keep at -80°C for 50 min., or at -20°C overnight.
4. Spin 40 min. in refrigerated microcentrifuge at 11000 rpm. Carefully remove the supernatant.
5. Wash pellet with 70% cold ethanol, add slowly ~200 μ l of cold 70% ethanol to the wall of the tube, invert the tube and spin for 10min in refrigerated microcentrifuge at 11000 rpm.
6. Remove supernatant, wipe the tube walls carefully and dry the pellet at 37°C for ~20 min.
7. Dissolve the pellet in 10-20 μ l TE buffer.
8. Incubate the tube in water bath at 37°C for 20 min to completely dissolve the pellet. Mix well.
9. Verify size of fragment by submitting specimen to gel electrophoresis using a 1% agarose gel to confirm that the product band has the expected size (small gel casting tray 50 ml, larger tray 150 ml + add EtBr prior to pouring the gel - ~4-6 μ l of 10mg/ml stock EtBr). Allow gel to cascade for 30-40 mins for a small gel, 45-60 min for a large gel, run at 85-90 volts.
10. In the marker lane, use appropriate size markers (ie 2 μ l of 100bp High Mass Ladder, 3 μ l of Gel Pilot 100 bp ladder or 0.5 μ l of 100 bp DNA ladder Invitrogen +1 μ l 6x dye + sterile water to complete 6 μ l. In the test DNA wells, use 6 μ l of DNA product (dye is part of GoTaq Green Master Mix)
11. Annotate the DNA vial with ID, concentration, date, tech initials, and buffer type.
12. Store DNA at -20°C.

Revised April 15, 2008 (LG/MS/SH)

Approved on _____ by _____

Read and understood by laboratory personnel

Name

Date

VI. DNA Cloning – Protocol 8

Amplification of DNA Sequences

I. Reagents

1. REPLI-g Midi Kit (Qiagen Cat. No. 150045)
2. Human Control Kit (Qiagen Cat. No. 150090)
3. Ethidium Bromide Stock Solution 10mg/ml
4. Agarose ultraPure (Life Technologies Cat. No. 15510-027)
5. 1 x Tris-Acetate Buffer (TAE) – Dilute 1 part 50x stock in 49 parts Milli-Q water
Prepare 50x stock buffer as follows: 242.0 g Tris base + 57.1 mL Glacial Acetic Acid +100 mL 0.5M EDTA pH 8.0 + Milli-Q water to complete 1L solution]
6. 6x loading dye
7. Markers for size of DNA bands: (a) 100bp High Mass Ladder; (b) 3 ul of Gel Pilot 100 bp ladder (Qiagen # 239035) or (c) 0.5 ul of 100 bp DNA ladder (Invitrogen # 15628-019)

II. Procedure

This protocol is optimized for BAC DNA templates. The optimal DNA concentration to be entered into the REPLI-g assay should be 100ng.

1. Prepare Stock Buffer DLB by adding 500 ul nuclease-free water to the tube. Mix thoroughly and centrifuge briefly. Note: Reconstituted Buffer DLB can be stored for 6 months at –20°C.
2. Prepare sufficient Buffer D1 (denaturation buffer) and Buffer N1 (neutralization buffer) for the total number of whole genome amplification reactions. Buffer D1 and Buffer N1 should not be stored longer than 3 months.

Preparation of Buffer D1 Component Volume*

Reconstituted Buffer DLB† 5 ul

Nuclease-free water 35 ul

Preparation of Buffer N1 Component Volume*

Stop Solution 8 ul

Nuclease-free water 72 ul

3. Clearly label a 200ul microcentrifuge tube and pipet 5 or **2.5** ul of template DNA into the tube.
The concentration of template DNA should be 100 ng. Lower starting concentrations of REPLI-g DNA may lead to inaccurate labeling reactions downstream, although there is evidence of successful amplification with as little as 11ng.
4. Add 5 or **2.5** ul Buffer D1 to the DNA for denaturation. Mix by vortexing and centrifuge briefly.
5. Incubate the samples at room temperature (15–25°C) for 3 min.
6. Add 10 or **5** ul Buffer N1 to the samples for neutralization. Mix by vortexing and centrifuge briefly.
7. Thaw REPLI-g Midi DNA Polymerase on ice. Thaw all other components at room temperature, vortex, then centrifuge briefly. The REPLI-g Midi Reaction Buffer may form a precipitate after thawing. The precipitate will dissolve by vortexing for 10 s.
8. Prepare a master mix on ice. Mix and centrifuge briefly.

Important: Add the master mix components in the order listed. After addition of water and REPLI-g Midi Reaction Buffer, briefly vortex and centrifuge the mixture before addition of REPLI-g Midi DNA Polymerase. The master mix should be kept on ice and used immediately upon addition of the REPLI-g Midi DNA Polymerase.

Preparation of Master Mix per specimen (5ul)

REPLI-g Midi Reaction Buffer 29ul

Preparation of Master Mix per specimen (2ul)

REPLI-g Midi Reaction Buffer **29ul**



Cytogenetics Core

Anschutz Medical Campus, L18-8401A
Mail Stop 8117 P.O. Box 6511 Aurora, CO 80045
Phones: 303-724-3147, 303-724-3148
Fax: 303-724-3889 www.uccc.info/cytogenetics

213

	Nuclease-free water	10ul
REPLI-g Midi DNA Polymerase 1ul	REPLI-g Midi DNA Polymerase	1ul

8. Add the 30 or **40** ul of the master mix to 20 or **10** ul of denaturated DNA (step 4).
9. Place the 200 ul microcentrifuge tube into the Applied BioSystems Thermal Cycler and select Run. Choose 'repli-g' and Start. The Thermal Cycler REPLI-g selection is pre-programmed with the following steps:
 - a. Incubate at 30°C for:
 - i. 16 hrs (recommended for non-repetitive sequence DNA)
 - ii. 6 or 8 hrs (recommended for repetitive sequence DNA)
 - b. Inactivate REPLI-g Midi DNA Polymerase by heating the sample for 3 min at 65°C.
 - c. Store amplified DNA at 4°C for short-term storage or -20°C for long-term storage.
10. Dilute the REPLI-g product 1:9 (REPLI-g product to Sterile Water)
11. Verify size of fragment by submitting specimen to gel electrophoresis using a 1% agarose gel to confirm that the product band has the expected size (small gel casting tray 50 ml, larger tray 150 ml + add EtBr prior to pouring the gel - ~4-6 ul of 10mg/ml stock EtBr). Allow gel to cascade for 30-40 mins for a small gel, 45-60 min for a large gel, run at 85-90 volts.
10. In the marker lane, use appropriate size markers (ie 2 ul of 100bp High Mass Ladder, 3 ul of Gel Pilot 100 bp ladder or 0.5 ul of 100 bp DNA ladder Invitrogen +1 ul 6x dye + sterile water to complete 6 ul. In the test DNA wells, use 6ul of DNA product (dye is part of GoTaq Green Master Mix)
12. Annotate the DNA vial with ID, concentration, date, and initials.
13. Store DNA at -20°C or proceed with labeling.

Revised April 15, 2008 (LG/MS/SH)

Approved on _____ by _____

Read and understood by laboratory personnel

Name

Date

IV. DNA Labeling - Protocol 3

DNA Direct Labeling by Nick-Translation

I. Reagents:

1. Nick Translation Kit (Abbott Molecular Cat. # 32-801300)
2. SpectrumGreen (Cat. # 30-803200), SpectrumOrange (Cat. # 30-803000) or SpectrumRed (Cat. # 30-803400) labeled nucleotides

II. Preparing the Reagents (Protect From Light):

1. 0.2 mM SpectrumGreen, SpectrumOrange or SpectrumRed. Add 10 ul of 1mM dUTP stock to 40 ul nuclease-free water
2. 0.1 mM dTTP. Add 10 ul of 0.3 mM dTTP to 20 ul nuclease-free water
3. 0.1 mM dNTP Mix. Mix together 10 ul each of 0.3 mM dATP, dCTP, and dGTP

III. Procedure (Protect from Light):

1. Prepare pure DNA. Labeling DNA not efficiently purified can result in poor incorporation of SpectrumGreen, SpectrumOrange or SpectrumRed labeled nucleotides and high background after hybridization.
2. Label a 0.5 ml microcentrifuge tube with the DNA ID, label to be used, and date. Place the tube on ice.
3. For each reaction, add the following to the labeled tube in the correct order:
 - a. 1 ug of DNA (x ul) and dH₂O (17.5 – x ul).
 - b. 5 ul Nick Translation Buffer
 - c. 2.5 ul of 0.2 mM SpectrumGreen, SpectrumOrange or SpectrumRed dUTP.
 - d. 5 ul of 0.1 mM dTTP.
 - e. 10 ul of 0.1 mM dNTP mix. Vortex briefly and pulse spin.
 - f. 10 ul of nick translation enzyme.

NOTE: Be sure to take an ice bucket to the freezer and place the nick-translation enzyme in it. Carry the bucket back to the workstation. Return the enzyme to the freezer immediately after use. Proper storage and handling procedures will ensure a long life for the enzyme mix.

4. Mix briefly and spin for 2 seconds (pulse spin) in a microcentrifuge.
5. Place the tube in a thermoblock, and incubate at 15°C for 3-20 hours, depending on the size of the DNA to be labeled and the desired size of the labeled fragments, according to the table below. For directions to use the thermoblock see Protocol IV. 4.

DNA	Initial Size of DNA	Final Size of Fragment	Incubation Time
2 ug	Plasmid DNA (~0.5-1kb of repeated sequences)	~50-100 bp	3 Hours
1 ug	~4.5 kb	~300 bp	4 Hours
1 ug	~7.9 kb	~300 bp	7 Hours



Cytogenetics Core

Anschutz Medical Campus, L18-8401A
 Mail Stop 8117 P.O. Box 6511 Aurora, CO 80045
 Phones: 303-724-3147, 303-724-3148
 Fax: 303-724-3889 www.uccc.info/cytogenetics

215

1 ug	~10 kb	~300 bp	8 Hours
1 ug	Cosmids (~40 kb)	~150-200 bp	12 Hours
1 ug	P1 DNA (~70-100 kb)	~300 bp	15 Hours
1 ug	BAC DNA (~100-200kb)	~300 bp	16 Hours: Cell Lines 18 Hours: Tissue Sect.
1 ug	YAC DNA (~200kb-1.5Mb)	~300 bp	20 Hours
1 ug	Genomic DNA (for CGH)	~300-1000 bp	3 Hours

6. Spin down labeled DNA for 2 seconds in a microcentrifuge and store at -20° C until needed (up to 2 years).

IV. Comments:

1. Before using the probes, it is convenient to determine the size of the labeled fragments (Protocol IV.P2). The probe can be left in this state or may be immediately precipitated for FISH (Protocol IV. P5).
2. For hybridization in formalin-fixed, paraffin-embedded tissue sections, probe fragments should be smaller and longer incubation times are needed. For BACs, 18 h. instead of 16 h. is optimal.
3. The amount of DNA used in the labeling reaction may be changed depending on the original size and the optimal final size. For instance, for 5 kb inserts, 2 ug of DNA may be introduced in the reaction and labeled for 8 hours; this is expected to provide more labeled DNA with the correct final size. In this case, the precipitation and hybridization is regularly performed taking the final volume of the labeled DNA as for 1 ug.

Revised April 8, 2008 (LG/SH)

Approved on _____ by _____

Read and understood by laboratory personnel

Name

Date

IV. DNA Labeling - Protocol 5

Probe Preparation for Hybridization

I. Reagents:

1. Cold 100% ethanol and 70% ethanol.
2. 3 M Na Acetate pH 5.4
3. 5 mg/ml salmon/herring sperm DNA.
4. 1 ug/ul Cot 1 DNA (Human, mouse, rat)
5. Hybridization Buffer: 65% formamide hybridization buffer for repetitive sequence; 50% formamide hybridization buffer for single sequences or commercial mix according to the manufacturer recommendations.

II. Procedure (Protect from Light):

1. Add to the 50 ul of labeling reaction
 - a. 50x the amount of labeled DNA (1ug) of salmon sperm (10 ug/ul) = 5 ul
 - b. 10x the amount of labeled DNA of Cot-1 DNA* (1 ug/ul) = 10 ul
 - c. 0.1x the volume of the reaction of 3M Na Acetate = 7.5 ul
 - d. 2-2.5x of the volume of reaction of 100% cold EtOH = ~180 ul

*** Cot-1 DNA is only added if the probe is not a repetitive sequence. In addition the reagent is species-specific (human, mouse, etc.) and its concentration may be adjusted to allow better signals**

2. Mix by inverting the tube and keep at -80°C for 30-60 min., or at -20°C overnight.
** Hold tube at 4 °C in cold room for 5 min before spinning to equilibrate.*
3. Spin 30 min. in refrigerated microcentrifuge at 11000 rpm. Remove the supernatant.
4. Wash pellet 2 times with 70% cold ethanol (slowly add ~200 ul of cold 70% ethanol to the wall of the tube, and invert the tube. If the pellet gets loose when you add the ethanol, it must be centrifuged again.
5. Wipe the tube walls carefully and dry the pellet at 56°C for ~15-30 min.
6. Redissolve the pellet in the desired amount of the appropriate hyb mix. (homebrew 65% formamide hybridization buffer for repetitive sequence; homebrew 50% formamide hybridization buffer for single sequences; for commercial hyb mixes follow manufacturer's instructions).
7. Incubate the tube in a water bath at 37°C for 15-30 min to completely dissolve the pellet. Mix well and store at -20°C until needed.

Comments: If the probe generates heavy background in the FISH preparations, it may help to precipitate the labeling reaction twice. In this case, at the first time only the labeled DNA, 3 M Na Acetate and 100% ethanol is used. Then, the pellet is dissolved in 100 ul of dH₂O, and the proper proportions of carrier and blocking DNA, 3 M Na Acetate and 100% ethanol are added.

III. For Hybridization with Mixture of Probes (for one 22 x 22 mm cover area)



Cytogenetics Core

Anschutz Medical Campus, L18-8401A
Mail Stop 8117 P.O. Box 6511 Aurora, CO 80045
Phones: 303-724-3147, 303-724-3148
Fax: 303-724-3889 www.uccc.info/cytogenetics

217

A. Combination of Unique Sequence (or Alpha Satellite) Probes

1. Mix the following components:
 - a. Selected amount of each labeled unique sequence DNA [about 100-150 ng for cosmid, PAC and BAC DNA, 100 to 200 ng for YAC DNA]
 - b. 10x of Cot-1 Human or the proper species (1 ug/ul) to labeled DNA (do not exceed 30 ug if mixing several probes)
 - c. 50x of salmon sperm DNA (5 ug/ul) or an amount that does not exceed a total of 15 ug of DNA, otherwise the hybridization solution will be too concentrated and the pellet will not dissolve in hybridization mix.
 - d. 0.1x the volume of the DNA solution of 3 M Na Acetate
 - e. 2-2.5x of the volume of reaction of 100% cold EtOH
2. Mix well and incubate at -80°C for 30 min.
3. Microspin at 4°C for 30 min. at 11,000 rpm. Remove the supernatant.
4. Carefully wipe the tube walls and dry the pellet at 56°C for 15-30 min.
5. Carefully rinse the pellet in 70% ethanol (slowly add ~200 ul of cold 70% ethanol to the wall of the tube, and invert the tube. If the pellet gets loose when you add the ethanol, it must be centrifuged again.
6. After the pellet is dry, add the desired amount of 50% formamide hybridization mix (minimum 10-15 ul) to dissolve the pellet. Incubate the tube at 37°C for 10 min. (it may be necessary to incubate in higher temperature or longer time). Mix by tapping with finger tip; spin 2 sec. in a microcentrifuge to pellet the droplets.
7. Store at -20°C or proceed for denaturation.

The steps below can be performed during the hybridization (see Protocols in section V. FISH).

8. Calculate the total volume needed for all slides (for example: 6 ul of this mixture is needed for a 15 mm dia. coverslip). Ratio of probe to dH₂O to 50% formamide hybridization buffer or LSI Hybridization Buffer is 1.0 ul probe to 2.0 ul dH₂O to 7.0 ul LSI Hybridization Buffer. Adjust the volumes accordingly for different coverslips.
9. Denature the probe mixture at 75-80°C for 5-10 min. (place the tube tightly closed, in a bubble rack and float in a water bath at 80°C for 10 min.).
10. Chill on ice for about 2-5 min. to condense vapor inside the tube. Pulse spin to collect the droplets.
11. Incubate the probe mixture in a water bath at 37°C for 20-30 min. for pre-annealing of the Cot-1 repetitive sequences (if applicable).
12. Chill the mixture on ice for 2-5 min. Pulse spin and keep on ice until use.

B. Combination of Probes with Repetitive and Unique Sequences

1. Proceed with steps 1 to 7 of III.A for repetitive sequence probes (**EXCEPT** that no Cot-1 DNA is added and 65% formamide hybridization buffer is used).



Cytogenetics Core

Anschutz Medical Campus, L18-8401A
Mail Stop 8117 P.O. Box 6511 Aurora, CO 80045
Phones: 303-724-3147, 303-724-3148
Fax: 303-724-3889 www.uccc.info/cytogenetics

218

2. Prepare unique sequence probes as defined in III.A.

The steps below can be performed during the hybridization (see Protocols in section V. FISH).

3. After blocking of the repetitive sequences of the unique sequence DNA, mix the appropriate amount of repetitive and unique sequence probes along with dH₂O and LSI Hybridization Buffer if necessary to obtain the required volume of the final mixture.
4. Denature the probe mixture at 75-80°C for 5-10 min and chill on ice until use.

Revised April 8, 2008 (LG/SH)

Approved on _____ by _____

Read and understood by laboratory personnel

Name

Date