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

Programa de Pós-Graduação em Genética

Lucimari Bizari Di Cézar

Estudo da amplificação, expressão e localização dos oncogenes *CCND1* e *HER-2/neu* nos territórios interfásicos cromossômicos em tumores de esôfago e estômago

Tese apresentada para obtenção do título de Doutor em Genética.

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Epígrafe

Epígrafe

"Bom mesmo é ir a luta com determinação
Abraçar a vida e viver com paixão,
Perder com classe e vencer com ousadia,
Pois, o triunfo pertence a quem se atreve
E a vida é muito para ser insignificante."
(Charles Chaplin)

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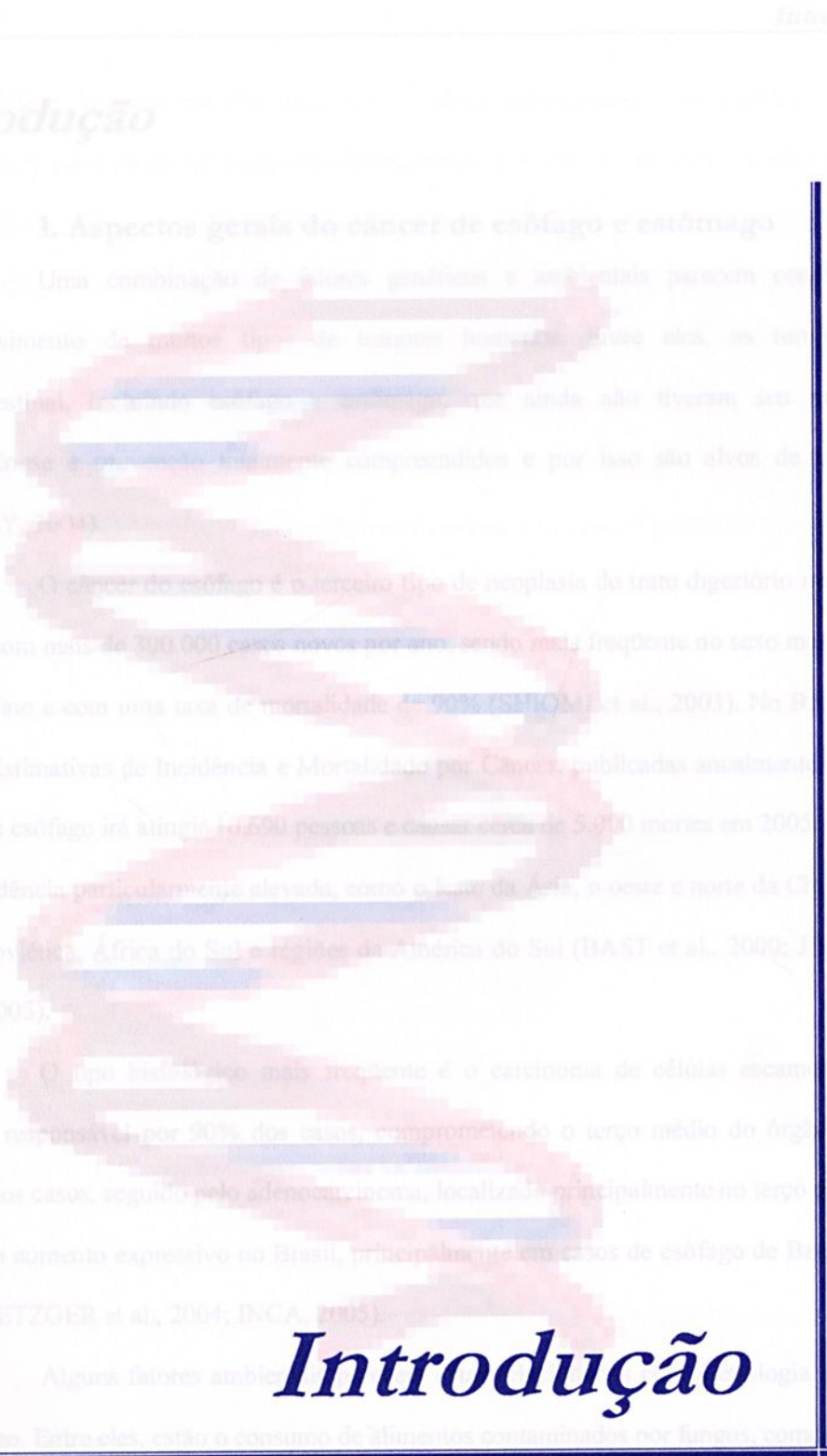
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Introdução

Introdução

1. Aspectos gerais do câncer de esôfago e estômago

Uma combinação de fatores genéticos e ambientais parecem contribuir para o desenvolvimento de muitos tipos de tumores humanos. Entre eles, os tumores do trato gastrointestinal, incluindo esôfago e estômago, que ainda não tiveram seu mecanismo de carcinogênese e prevenção totalmente compreendidos e por isso são alvos de vários estudos (HEAVEY, 2004).

O câncer do esôfago é o terceiro tipo de neoplasia do trato digestório mais comum no mundo, com mais de 300.000 casos novos por ano, sendo mais freqüente no sexo masculino do que no feminino e com uma taxa de mortalidade de 90% (SHIOMI et al., 2003). No Brasil, de acordo com as Estimativas de Incidência e Mortalidade por Câncer, publicadas anualmente pelo INCA, o câncer de esôfago irá atingir 10.690 pessoas e causar cerca de 5.000 mortes em 2005. Existem áreas com incidência particularmente elevada, como o leste da Ásia, o oeste e norte da China, Japão, Irã, União Soviética, África do Sul e regiões da América do Sul (BAST et al., 2000; JIN et al., 2004; INCA, 2005).

O tipo histológico mais freqüente é o carcinoma de células escamosas (CCE) do esôfago, responsável por 90% dos casos, comprometendo o terço médio do órgão em cerca de metade dos casos, seguido pelo adenocarcinoma, localizado principalmente no terço distal, que vem tendo um aumento expressivo no Brasil, principalmente em casos de esôfago de Barret (JIN et al., 2004; METZGER et al., 2004; INCA, 2005).

Alguns fatores ambientais parecem estar relacionados com a etiologia das neoplasias de esôfago. Entre eles, estão o consumo de alimentos contaminados por fungos, como o *Aspergillus*, as dietas ricas em nitritos e nitrosaminas, a deficiência nutricional crônica das vitaminas A e C e de alguns membros do complexo B ou de oligoelementos como o zinco e o molibdênio (BAST et al., 2000) e o abuso crônico de álcool e do fumo (BAST et al., 2000; GUILEM et al., 2000; VERLAG



et al., 2001). Algumas bebidas alcoólicas contém hidrocarbonetos aromáticos policíclicos e nitrosaminas, além de outros compostos mutagênicos. Portanto, a deficiência nutricional associada ao alcoolismo pode contribuir para o processo carcinogênico. Também estão relacionadas com seu desenvolvimento a predisposição genética e a presença de lesões benignas, como a acalasia e o esôfago de Barret citado acima (COTRAN et al., 1999; LEHRBACH et al., 2004).

O câncer de estômago é o terceiro tipo mundialmente mais comum em incidência e mortalidade e duas vezes mais freqüente em homens do que em mulheres, principalmente em idosos (PARKIN, 2001; INCA, 2005). As estatísticas mundiais mostram uma distinta variação geográfica, com taxas elevadas no Japão, leste da Europa, Ásia Oriental, América Central e América do Sul, principalmente no Chile e Costa Rica e taxas mais baixas em países desenvolvidos (AICR, 1997). A estimativa da incidência e mortalidade de câncer de estômago no Brasil, para 2005, é de 23.145 casos novos e cerca de 11.000 óbitos (INCA, 2005). O prognóstico é extremamente pobre, com valores de sobrevida de cinco anos ocorrendo em 5-15% dos indivíduos afetados em países desenvolvidos (BRIEN et al., 1998). Tal fato reflete que a maioria dos casos são diagnosticados em estágio avançado e, mesmo após gastrectomia, a taxa de recorrência da doença é de 80% (DE-VITA et al., 1997).

As principais lesões que podem acometer o estômago incluem a gastrite aguda e a crônica, as erosões e as ulcerações gástricas agudas, as úlceras pépticas e as neoplasias benignas e malignas. Dentre as neoplasias malignas do estômago, o adenocarcinoma derivado do epitélio é o mais comum e importante, com freqüência de 90-95% dos casos (COTRAN et al., 1999), sendo classificados com base em aspectos histopatológicos e clínicos em dois tipos: intestinal e difuso (LÁUREN et al., 1996; TAHARA, 1998, 2004).

As lesões tipo intestinal são mais freqüentes, bem diferenciadas, dependentes de fatores ambientais e associadas com a presença de lesões pré-neoplásicas, como gastrite crônica, atrofia gástrica, metaplasia intestinal e displasia (HOHENBERGER; GRETCHEL, 2003; TAHARA, 2004). São encontradas predominantemente em homens e em indivíduos idosos. O tipo



difuso é pouco diferenciado, tem prognóstico ruim e não está associado a lesões pré-cancerosas (SEGAL et al. 2001). É um pouco mais freqüente em mulheres e em pacientes jovens e tem uma alta ocorrência familial, principalmente entre indivíduos com tipo sanguíneo "A" (DE-VITA et al., 1997).

Dados epidemiológicos sugerem que a dieta assim como outros fatores ambientais têm um papel importante na etiologia do câncer gástrico, como consumo elevado de sal, nitratos, nitritos e álcool, cirurgia gástrica anterior, infecção pela bactéria *Helicobacter pylori*, bem como lesões benignas do tipo gastrite atrófica crônica, úlcera gástrica, metaplasia intestinal e displasia e predisposição familial (DE VITA et al., 1997; SONG et al., 2003; CORREA et al., 2004; HEAVEY et al., 2004). Acredita-se que a ação dos compostos nitrogenados e seus metabólitos esteja relacionada com a inibição de vitamina C e de outros antioxidantes que protegem o DNA (DE-VITA et al., 1997; SEGAL et al., 2001). De modo contrário, os carotenóides, as vitaminas C e E, o folato e o selênio presentes em frutas e vegetais frescos estão relacionados com o efeito protetivo da mucosa gástrica contra danos oxidativos (AICR, 1997; SEGAL et al., 2001; HEAVEY et al., 2004).

A bactéria *H. pylori,* Gram-negativa, encontrada no estômago humano, tem sua presença reconhecida como o agente desencadeante de diferentes alterações gástricas, como inflamação crônica assintomática, dispepsia, úlcera, adenocarcinomas e linfomas (HIRATA et al., 2001; SONG et al., 2003). Contudo, a prevalência da infecção nas populações não parece estar correlacionada com as taxas de câncer gástrico. Por exemplo, populações na África apresentam prevalência elevada de infecção pela *H. pylori*, mas baixa incidência de câncer gástrico (HOLCOMBE, 1992). Portanto, a infecção por *H. pylori* não é a única causa para o desenvolvimento do câncer, cujo risco é de apenas 1% entre os indivíduos infectados.

O conhecimento sobre o papel da *H. pylori* na tumorigênese gástrica ainda é incompleto (POLAT et al., 2002; HOHENBERGER; GRETCHEL, 2003; HEAVEY, 2004). Alguns mecanismos já foram propostos, como a desregulação do ciclo celular, formação de adutos no DNA, geração de radicais livres, danos oxidativos, alterações em fatores de crescimento e citocinas

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e efeitos sobre a secreção gástrica (SEGAL et al., 2001; POLAT et al., 2002; SEPULVEDA et al., 2002; HEAVEY, 2004). Os efeitos inflamatórios da infecção pela *H. pylori* têm sido relacionados ao câncer gástrico devido à proliferação celular aumentada e produção de radicais livres mutagênicos e compostos N-nitrosos (MOSS, 1999), acarretando mutações que podem ser imortalizadas no epitélio gástrico (CORREA; MILLER, 1998).

2. Alterações genéticas envolvidas na carcinogênese do esôfago e estômago

O desenvolvimento do câncer de esôfago é um processo de múltiplas etapas e ocorre de forma progressiva. Um indicador precoce é o aumento de proliferação de células epiteliais, levando a hiperplasia, displasia e ao carcinoma *in situ*. O processo de tumorigênese em nível celular está relacionado com alterações em genes responsáveis pelo controle do ciclo celular, ou seja, genes de proliferação, diferenciação e apoptose. Neste processo, muitas alterações genéticas estão envolvidas, incluindo mutações nos genes supressores de tumor *TP53, WAF1/CIP1, ARF e INK4a*; amplificação dos oncogenes *CCND1* e *c-MYC;* além de alterações em genes de apoptose, como o *BCL-2* e o *BAX*, que também interagem com supressores de tumor no controle da proliferação celular (LEHRBACH et al., 2004).

Alterações nos genes *TP53* e WAF1/CIP1 estão envolvidos com o estágio inicial da carcinogênese esofágica, não sendo correlacionadas com invasão tumoral, metástases ou invasão de linfonodos (NITA et al., 1999; BAHL et al., 2000; PUTZ et al., 2002; LEHRBACH et al., 2004). Enquanto, alterações em genes que controlam a apoptose, como *BAX* e *BCL-2,* parecem ocorrer principalmente em tumores mais avançados, portanto sendo um evento tardio na carcinogênese do esôfago (LEHRBACH et al., 2004).

Além desses genes, alterações citogenéticas relacionadas com os carcinomas de esôfago incluem alguns cromossomos e regiões cromossômicas específicas, como ganho dos cromossomos 7, 11 e 17; da região 12p e perdas 3pq, as quais estão relacionadas com um

prognóstico pior e ocorrem tardiamente (SHIOMI et al., 2003; KWONG et al., 2004; MANOEL-CAETANO et al., 2004). O ganho da região 8q está relacionado com tumores mais invasivos e as perdas em 1p e 16p com invasão linfática (NOGUCHI et al., 2003).

Nos carcinomas de células escamosas e adenocarcinomas de esôfago, e nos tumores de cabeça e pescoço e de laringe, ocorre amplificação da região 11q13 (*CCND1),* que parece indicar prognóstico ruim, incluindo invasão local, metástases em linfonodos, estágio avançado da doença e aumento no risco de recorrência (ISHIZUKA et al., 2002; OZAWA et al., 2002; MILLER et al., 2003; MYIAMOTO et al., 2003; NADAL et al., 2003; MANOEL-CAETANO et al., 2004; METZGER et al., 2004).

Todos esses eventos de interação entre os genes e suas funções durante o processo carcinogênico têm contribuído para o entendimento das alterações genéticas que levam ao desenvolvimento do CCE do esôfago e poderão auxiliar no diagnóstico e prognóstico desses tumores de um modo mais preciso (LEHRBACH et al., 2004), assim como para o direcionamento de terapias com alvos moleculares.

Em câncer gástrico, estudos citogenéticos e moleculares têm identificado ganhos e perdas de cromossomos, amplificações gênicas e mutações em oncogenes e genes supressores de tumor em um processo de múltiplas etapas, assim como ocorre em carcinoma de esôfago. Entretanto, existe uma combinação diferente dessas alterações nos dois tipos histológicos de câncer gástrico, indicando vias carcinogênicas distintas para o tipo difuso e o intestinal (TAHARA, 2004). No carcinoma tipo intestinal, parece ocorrer a seqüência hiperplasia - metaplasia – adenoma – carcinoma, na qual a infecção com *H. pylori* pode ser um forte fator para a passagem de hiperplasia para metaplasia intestinal. Durante essa fase começam a ocorrer erros de replicação e instabilidade genética como, hipermetilação do DNA e mutações em *TP53* que se acumulam em pelo menos 30% das metaplasias intestinais. Após essa fase, outras alterações como mutações em *APC,* LOH em 1q, perda em p27, expressão reduzida de fatores de crescimento e amplificação do oncogene *HER-*

2/neu estão freqüentemente relacionados com o câncer gástrico do tipo intestinal em estágio avançado (TAHARA, 2004).

Nos carcinomas gástricos do tipo difuso, as alterações mais freqüentes são mutações em 17p, perda de heterozigosidade em *TP53,* mutações e perdas no locus da *E-caderina* (KOO et al., 2000; STAMOULI et al., 2002; TAHARA, 2004). Além dessas alterações, podem ocorrer também amplificação dos oncogenes *K-SAM, c-MET* e perdas de p27 nos estágios mais avançados da doença, resultando em progressão e metástases (SEGAL et al. 2001; PENG et al., 2003; TAHARA, 2004).

Diversos estudos têm demonstrado que alguns oncogenes apresentam-se amplificados em um número significativo de tumores humanos, como *CCND1*, *c-MET*, c-*MYC*, *HER-2/neu, EGFR* e *MDM2*. Entre eles, o *CCND1* e o *HER-2/neu* parecem estar mais freqüentemente amplificados nos tumores de esôfago (MANOEL-CAETANO et al., 2004; SHIOMI et al., 2004) e de estômago (TAKEHANA et al., 2002; DAHLBERG et al., 2004).

O oncogene *CCND1,* localizado em 11q13, apresenta-se amplificado em carcinomas de mama, bexiga, cabeça e pescoço, pulmão e carcinomas de células escamosas de esôfago (HUANG et al., 2002; ISHIZUKA et al., 2002; MIYAMOTO et al., 2003). Similarmente, a expressão aumentada de seu produto, a proteína ciclina D1, também ocorre nesses casos, mas pode estar ou não relacionada com a amplificação gênica (MIYAMOTO et al., 2003; MORENO-BUENO et al., 2003; EWEN; LAMB, 2004).

Nos carcinomas de células escamosas e adenocarcinomas de esôfago, e nos tumores de cabeça e pescoço e de laringe, a amplificação do oncogene *CCND1* também está relacionada com prognóstico ruim, incluindo invasão local, metástases em linfonodos, estágio avançado da doença e aumento no risco de recorrência (ISHIZUKA et al., 2002; OZAWA et al., 2002; MILLER et al., 2003; MIYAMOTO et al., 2003; NADAL et al., 2003). Tanto a amplificação do oncogene como a expressão aumentada da proteína ciclina D1 parecem também estar relacionadas com resistência do tumor ao tratamento quimioterápico em câncer de esôfago (NAGASAWA et al., 2001).

O *c-ERBB-2,* também conhecido como *HER-2/neu*, está localizado na região 17q11.2-q12 (YAMAMOTO et al., 1986) e constitui outro oncogene freqüentemente amplificado em tumores de origens variadas, como mama, ovário, bexiga, estômago e pulmão (TAKEHANA et al., 2002; LEAR-KAUL et al., 2003) sendo um potencial marcador prognóstico em diversos tipos de tumores, incluindo mama e ovário (ORMANDY et al., 2003; EWEN; LAMB., 2004). Nos tumores gástricos, a expressão elevada da proteína Her-2/neu ou p185, está relacionada com presença de metástases (NESSLING et al., 1998; VIDGREN et al., 1999; VARIS et al., 2002) e com evolução no tipo intestinal (BECKER et al., 2000).

Conforme relatado, a ativação de vários oncogenes por meio da amplificação gênica está relacionada com a progressão de vários tipos de tumores, inclusive os de esôfago e de estômago (SHIOMI et al., 2003; RIBAL et al., 2004; SUNPAWERAVONG et al., 2005). Diversos estudos têm evidenciado que os pacientes com amplificação gênica e polissomia apresentam um prognóstico pior quando comparado com aqueles sem amplificação. Desse modo, o ganho desses oncogenes desempenha um importante papel na progressão dos carcinomas, podendo aumentar os riscos de metástases e de tumores mais agressivos. Contudo, a evidência citogenética de amplificação gênica não indica necessariamente a expressão de todas as cópias do gene envolvido. Por esse motivo, são necessários estudos que avaliem tanto a amplificação gênica como a expressão protéica.

3. O estudo dos territórios cromossômicos e expressão gênica

A estrutura da cromatina e sua regulação epigenética via modificações estruturais desempenham um papel importante na expressão gênica, evidenciando que a arquitetura nuclear e a organização espacial do genoma participam na regulação dessa expressão (PARADA; MISTELI, 2002). Conforme vários estudos, o local ocupado pelos cromossomos e seus elementos genéticos dentro do núcleo, domínios ou territórios cromossômicos não é casual (CROFT et al., 1999; VERSCHURE et al., 1999; FALK et al., 2002). Entretanto, os princípios desse arranjo e as

manifestações funcionais ainda não são conhecidos (FALK et al., 2002), mas parece que existe uma distribuição radial da cromatina no núcleo interfásico que depende do tamanho, densidade gênica e do tempo de replicação (TANABE et al., 2002).

A técnica de hibridização *in situ* fluorescente (FISH) vem sendo empregada em muitos estudos de arquitetura nuclear, empacotamento e organização da cromatina e para a determinação de territórios cromossômicos (CROFT et al., 1999; BARTOVA et al., 2000; WILLIAMS et al., 2003).

O território cromossômico é uma região discreta do volume nuclear ocupada pelo cromossomo durante o ciclo celular (CREMER et al., 1993; VISSER; ATEN, 1999). Os mecanismos de regulação e expressão gênica parecem estar relacionados não apenas com uma seqüência linear de DNA, mas com uma estrutura tridimensional que afeta a regulação gênica durante o ciclo celular (EDELMANN et al., 2001; PARADA et al., 2004). Esses dados têm sido confirmados por estudos recentes sobre organização nuclear que evideciaram uma correlação entre a localização periférica de genes dentro do território interfásico e seu estado transcricional (RAGOCZY et al., 2003).

A alta densidade de genes durante o processo de empacotamento da cromatina no espaço nuclear fornece uma base para o entendimento da organização espacial do genoma. A marcação de sítios de transcrição e sua localização por microscopia eletrônica demonstraram que a transcrição ocorre na superfície das fibras cromatínicas altamente condensadas. A diferenciação da cromatina rica em gene (banda R positiva) e deficiente em gene (banda G positiva) é mantida durante a intérfase e pode ser distinguida no estudo dos territórios cromossômicos. Desse modo, parece haver também uma distribuição cromossômica diferencial no espaço intra-nuclear, onde a cromatina rica em genes parece localizar-se no interior do núcleo quando comparada com a deficiente em genes, que parece estar presente na periferia nuclear (HENDZEL et al. 2001).

Nas células de galinha, cada território cromossômico também ocupa um local determinado dentro do núcleo que é dependente de seu tamanho, da densidade gênica e do tempo de

replicação. As regiões ricas em genes e os microcromossomos que replicam mais precocemente localizam-se no interior do núcleo, enquanto as regiões deficientes em genes e os macrocromossomos que replicam tardiamente localizam-se na periferia do núcleo (TANABE et al., 2002). Da mesma forma, em humanos, os cromossomos 18 e 19 de tamanhos semelhantes também mostram posição diferente dentro do núcleo interfásico, onde as regiões ricas em genes que replicam precocemente estão localizadas no centro nuclear (cromossomo 19) e as regiões pobres em genes e que replicam mais tardiamente estão na periferia (cromossomo 18) (TANABE et al., 2002; CREMER et al., 2004). Similarmente, alguns genes como *MYC*, *BCL* e o *locus* da imunoglobulina, freqüentemente translocados em linfoma de células B parecem estar preferencialmente próximos no núcleo interfásico, sugerindo que as translocações que ocorrem nesse tipo tumoral também são devidas à localização não casual dos genes dentro do núcleo (ROIX et al., 2003)

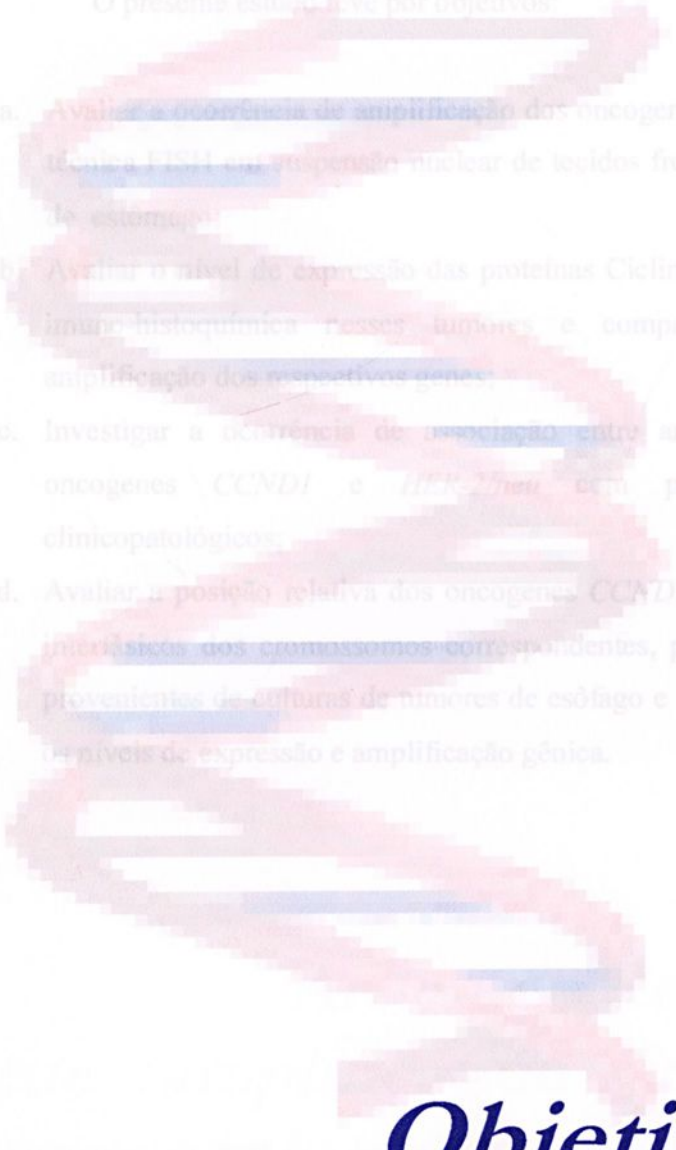
Zirbel et al. (1993) observaram, em diferentes células de mamíferos utilizando uma combinação de imunodetecção e FISH, que os transcritos de RNA e a maquinaria de *splicing* ocupavam geralmente a periferia dos territórios cromossômicos. Esses achados levaram estes autores a propor o modelo de Compartimentalização de Domínios Intercromossômicos (ICD), segundo o qual os genes ativos estão posicionados na periferia dos territórios cromossômicos interfásicos, onde ficam mais acessíveis à maquinaria transcricional, e os RNAs recém-sintetizados são depositados diretamente no espaço intercromatínico. Posteriormente, outros estudos corroboraram esses achados. Park; De Boni (1998) verificaram, em câncer de mama, que o gene *HER-2/neu* estava localizado preferencialmente na periferia do domínio do cromossomo 17, podendo representar um mecanismo de controle fundamental na expressão gênica. Do mesmo modo, quando células leucêmicas foram estimuladas para a diferenciação em granulócitos por dimetilsulfoxido (DMSO) ou ácido retinóico, o gene c-*MYC*, localizado no cromossomo 8, foi visualizado pela técnica de FISH na periferia nuclear (BARTOVA et al., 2000). Volpi et al. (2000), utilizando sondas para toda extensão do cromossomo 6 e de seqüência única para os genes do Complexo Principal de Histocompatibilidade (MHC), mostraram que os genes desse complexo,

cm 1 2 3 4 5 6 7 unesp 11 12 13 14 15 16 17 18

quando ativos, localizavam-se preferencialmente na periferia do domínio do cromossomo 6. Esses dados sugerem, portanto, que o posicionamento de seqüências ativas na periferia dos territórios cromossômicos está relacionado com sua transcrição (VERSCHURE et al., 1999; CHEVRET et al., 2000). Contudo, Mahy et al. (2002) estudando algumas seqüências gênicas (*RCN, PAXNEB, WT1* e *PAX6*) ativas em tumor de Wilm's pela técnica FISH, observaram que os genes transcricionalmente ativos não estavam localizados preferencialmente na periferia dos territórios cromossômicos. Para explicar esse achado, propuseram que ocorre uma mobilidade das proteínas e enzimas relacionadas com a transcrição gênica dentro de compartimentos subcromossômicos, não necessitando de uma posição preferencial periférica ou externa de tais genes.

No presente estudo, a técnica de FISH foi utilizada em câncer de esôfago e estômago para investigar se os genes *CCND1* e *HER-2/neu*, em situações de amplificação e expressão elevada, posicionam-se em locais preferenciais dos núcleos interfásicos. Os resultados, poderão ampliar os conhecimentos sobre posição e função da cromatina e contribuir para o entendimento da progressão tumoral.

Objetivos



Objetivos

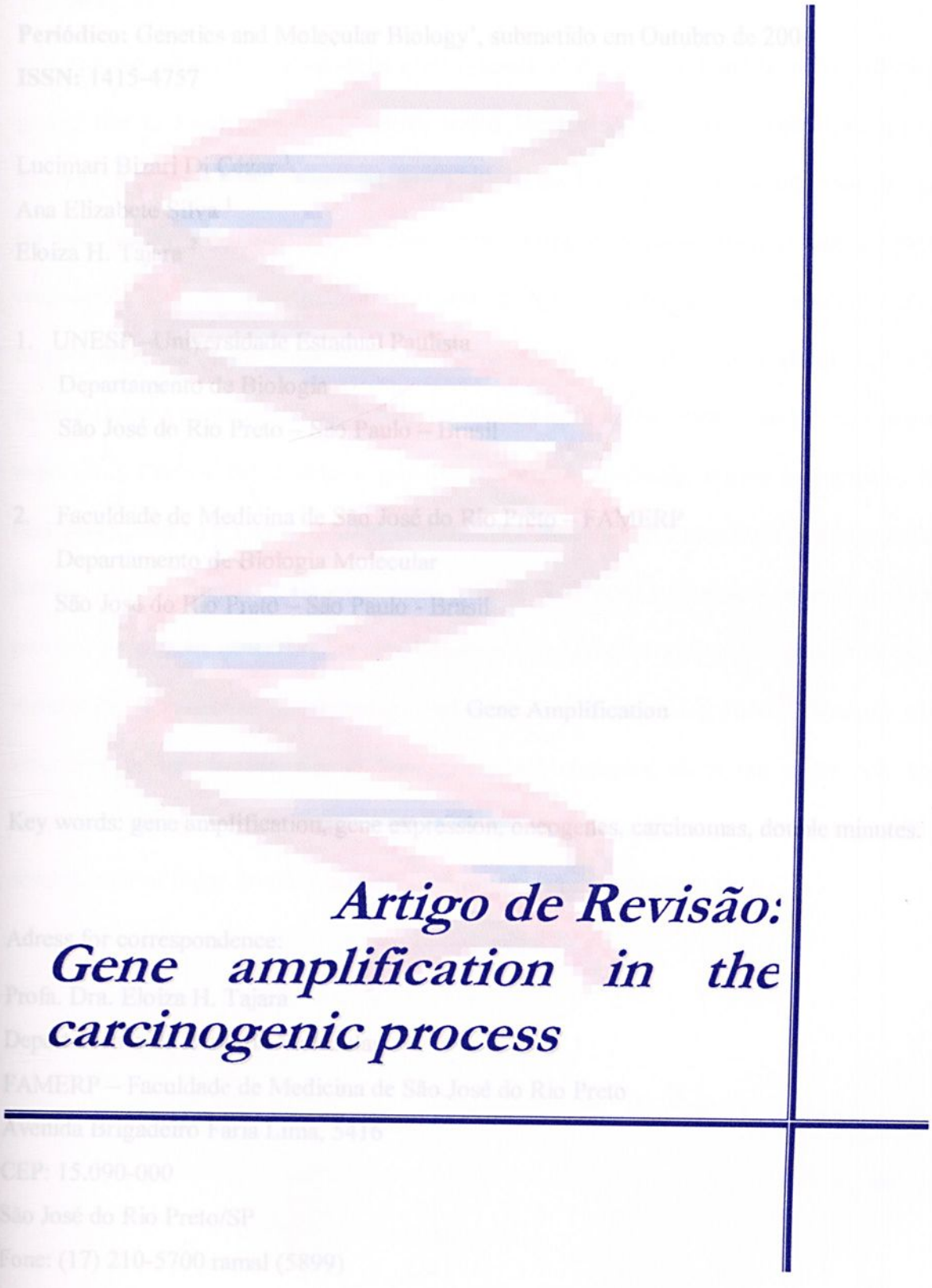
Objetivos

O presente estudo teve por objetivos:

a. Avaliar a ocorrência de amplificação dos oncogenes *CCND1* e *HER-2/neu*, pela técnica FISH em suspensão nuclear de tecidos frescos de tumores de esôfago e de estômago;
b. Avaliar o nível de expressão das proteínas Ciclina D1 e Her-2/neu por técnica imuno-histoquímica nesses tumores e comparar com a ocorrência de amplificação dos respectivos genes;
c. Investigar a ocorrência de associação entre amplificação e expressão dos oncogenes *CCND1* e *HER-2/neu* com parâmetros demográficos e clinicopatológicos;
d. Avaliar a posição relativa dos oncogenes *CCND1* e *HER-2/neu* nos territórios interfásicos dos cromossomos correspondentes, pela técnica FISH em células provenientes de culturas de tumores de esôfago e de estômago e comparar com os níveis de expressão e amplificação gênica.

Artigo de Revisão:
Gene amplification in the carcinogenic process

cm 1 2 3 4 5 6 7 unesp 11 12 13 14 15 16 17 18



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GENE AMPLIFICATION IN THE CARCINOGENIC PROCESS

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Gene Amplification

Key words: gene amplification, gene expression, oncogenes, carcinomas, double minutes.

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GENE AMPLIFICATION IN THE CARCINOGENIC PROCESS

I. ABSTRACT

Gene amplification comprises the expansion of the gene number in the cell genome giving rise to karyotype abnormalities called Double Minutes (DM) and Homogeneously Staining Regions (HSR). The DM and HSR forms have been widely observed in human tumors, but they can also play a major role during embryonic development, as they are responsible for the programmed increase of gene expression. The etiology of gene amplification during the process of carcinogenesis is not yet completely understood, but it can be considered a result of genetic instability. Gene amplification leads to an increase in protein expression, thereby being able to provide a selective advantage during cell growth. Some oncogenes, such as *CCND1, c-MET, c-MYC, HER-2/neu, EGFR,* and *MDM2*, are amplified in human tumors, they can be associated with an increased expression of their respective proteins or not. In general, gene amplification is associated with more aggressive tumors, metastases, resistance to chemotherapy and a decrease in the period during which the patient stays free of the disease. So, in this review, we comment about the major role in the progression of carcinomas, forming genetic markers and possible therapeutic targets for the development of drugs for the treatment of some types of tumors.

GENE AMPLIFICATION

Gene amplification can be defined as an expansion of the number of copies of a gene in the cell genome. This process occurs by replication of the genomic DNA, giving rise to karyotype abnormalities called Double Minutes (DM) and Homogeneously Staining Regions (HSR) (Bast *et al.,* 2000; Simon *et al.,* 2002).

Double minutes (DM) are extrachromosomal structures of circular DNA containing 1 to 2 million base pairs, which replicate autonomously about once every cell cycle and, since centromeres are absent, segregate at random to the daughter cells. The homogeneously staining regions (HSR), on the other side, are intrachromosomal segments forming large genomic regions (Hellman *et al.,* 2002), which display no chromosome banding pattern when submitted to the G-banding technique, but can be visualized by FISH. Both these structures have amplified genomic DNA, containing hundreds of copies of one or more genes, frequently oncogenes (Bast *et al.,* 2000).

Gene amplification plays a major role during development, when it is responsible for the programmed increase of gene expression. In amphibian oocytes, for example, where the need for proteins is high, an amplification of rRNA (ribosomal RNA) genes occurs. This process produces between two thousand and one million copies of rRNA per oocyte. Another example is found in *Drosophila* oocytes, which display amplification of genes responsible for chorionic proteins (Cooper, 2000). Another non-pathologic condition involving gene amplification was recently studied by Hastings & Rosenberg (2002). These authors observed that, under certain conditions of environmental stress, such as lactose deficiency, the bacterium *E. coli* exhibits amplification of the chromosome region containing the *LAC* allele and a higher production of the enzyme lactase, enabling the bacteria to grow even in a restrictive environment.



DM and HSR gene amplification has been widely observed in human tumors, where it acts as one of the oncogene-activating genetic mechanisms (Bast *et al.,* 2000; Imoto *et al.,* 2001) and frequently suggests an aggressive behavior of the tumor and a poor prognosis (Ohta *et al.,* 2001; Ethier *et al.,* 2003).

The etiology of gene amplification in cancer is not yet completely understood, but it can be considered a result of genetic instability. Cytogenetic studies in rats involving genes for drug resistance have indicated that in tumor cells there is a frequent occurrence of chromosome breaks followed by fusion of distant segments and the consequent formation of bridges during anaphase (Hellman *et al.,* 2002). This process is particularly common in certain fragile sites, which are unstable regions of the chromosomes, prone to breaks or gaps during cell division. Hypoxia, a powerful inducer of fragile sites, is thought to facilitate the fusion of DM and their reintegration into the chromosomal fragile sites, producing HSR (Ishizuka *et al.,* 2002).

Gene amplification has been demonstrated in several types of tumors, but their relationship with carcinogenesis and tumor progression is still not clear. This process leads to an increase in gene expression, which can confer a selective advantage during the early stages of the neoplastic process. However, amplification may also occur as a late event of tumorigenesis (Tsujimoto *et al.,* 1997; Bast *et al.,* 2000; Sarasin, 2003).

MAIN AMPLIFIED GENES IN HUMAN NEOPLASIAS

Several studies have shown that some oncogenes, such as *CCND1*, *c-MET*, c-*MYC*, *HER-2/neu, EGFR* and *MDM2*, are amplified in a significant number of human tumors. These and other oncogenes rarely become amplified alone, but rather present as large amplicons with multiple copies of several genes, making it difficult to establish which of

these genes provide a proliferative advantage (Nakakuki *et al.,* 2002; Simon *et al.,* 2002; Ethier, 2003).

An amplicon located in region 11q13 includes the oncogene *CCND1* and another two genes, *INT-2* and *HST-1*. This group of genes is frequently amplified in breast, bladder, head and neck, lung and esophageal squamous cell carcinomas (Huang *et al.,* 2002; Ishizuka *et al.,* 2002). Among these, *CCND1* is the most frequently amplified gene in tumor cells (Nagasawa *et al.,* 2001; Huang *et al.,* 2002; Miyamoto *et al.,* 2003). Overexpression of the Cyclin D1 protein also occurs in these cases, but it may or may not be related to gene amplification (Miyamoto *et al.,* 2003; Moreno-Bueno *et al.,* 2003).

Cyclin D1 is a 38kDa protein that belongs to the type cyclin D family, which includes cyclins D2 and D3 (Ewen & Lamb, 2004). It regulates the passage of cells in phase G1 to S in the cell cycle by phosphorylation of the pRb protein during phase G1, releasing the family of E2F transcription factors (Rabbani *et al.,* 2000; Brandau *et al.,* 2001; Vielba *et al.,* 2003) which, in turn, lead to the induction of the genes needed for the G1-S progression of the cell cycle (Ewen & Lamb, 2004). It acts by forming the cyclin D1-CDK complex, activating the specific CDKs (cyclin-dependent protein kinases – CDK4 e CDK6), thereby allowing the cell cycle to progress (Rabbani *et al.,* 2000; Saikawa *et al.,* 2001) (Figure 1).

Amplification of the gene encoding Cyclin D1 is seen in a variety of solid tumors, includig breast adenocarcinoma, squamous cell carcinoma of head and neck, esophageal and bladder cancer (Ewen & Lamb, 2004).

In bladder tumors, the overexpression of this protein is correlated with high-degree tumors and a short recurrence time. In patients with bladder cancer, there appears not to be any association between tumor invasion and decreased survival and overexpression of the Cyclin D1 protein (Adshead *et al.,* 1998). Amplification of the *CCND1* oncogene is also

seen in the early stages of these tumors, persisting during the advanced stages of the disease, but the exact mechanism leading to progression remains to be clarified (Watters *et al.,* 2002).

In esophageal squamous cell carcinomas and adenocarcinomas and in head and neck and larynx tumors, amplification of the *CCND1* oncogene is also related to a poor prognosis, including local invasion, metastases in lymph nodes, an advanced stage of the disease and an increased recurrence risk (Nagasawa *et al.,* 2001; Ishizuka *et al.,* 2002; Ozawa *et al.,* 2002; Nadal *et al.,* 2003; Miller *et al.,* 2003; Miyamoto *et al.,* 2003). Both the amplification of the oncogene and the overexpression of the D1 cyclin also seem to be related to the resistance of the tumor to chemotherapeutic treatment in esophageal cancer (Nagasawa *et al.,* 2001). In breast cancer, both gene amplification and overexpression of the protein are related to a shorter survival time, a shorter period free of the disease and a higher recurrence rate of the tumor (Ormandy *et al.,* 2003).

Another widely studied amplicon is located in region 7q31-q32 and includes the c-*MET* oncogene. This oncogene encodes the hepatocyte growth factor (HGF) receptor (Herynk *et al.,* 2003) and is one of the oncogenes most frequently associated with the development of gastric cancer, where it exhibits alterations such as amplification and overexpression of its protein (Nessling *et al.,* 1998). The amplification of this oncogene is correlated with the stage of the tumor, specifically of the diffuse type, as it is never found in early stages of carcinogenesis; it works as a marker of poor prognosis, providing progression and increase of metastases (Nessling *et al.,* 1998; Tsugawa *et al.,* 1998; Tahara, 2004).

Gene amplification and deregulation of the expression of the *c-MYC* oncogene located in region 8q24 are the main activating mechanisms of this oncogene in human tumors (Mai *et al.,* 2003; Abba *et al.,* 2004). Its protein (myc) enables the cell to enter the cell cycle, activating several genes, among which those encoding ornithine decarboxylase, phosphatase cdc25A and the transcription factor E2F (Zajac-Kaye, 2001; Mai *et al.,* 2003), and repressing



others, such as, for example, releasing protein p27 from the cdk2 complex by means of the activity of the cyclin E-cdk2 complex. Once released, protein p27 is sequestered by the cdk4-cyclin D complex, forming a new complex, cdk4-cyclinD-p27, that will phosphorylate pRb, releasing the transcription factor E2F and enabling the cell to perform the G1-S passage of the cell cycle (Zajac-Kaye, 2001). Overexpression of the c-myc protein can increase cell proliferation, impair differentiation and lead to an increase in cell apoptosis (Abba *et al.,* 2004). Usually, its expression decreases when the cell is leaving the cell cycle and during differentiation.

In several types of cancer, such as breast and hepatocellular (Ghani-Abdel *et al.,* 2002; Robanus-Maandag *et al.,* 2003), prostate, cervical (Abba *et al.,* 2004) and lung tumors (Zajac-Kaye *et al.,* 2001; Yakut *et al.,* 2003), amplification of *c-MYC* is correlated with protein overexpression and is frequently associated with more aggressive tumors.

In breast cancer, there is an association between *c-MYC* amplification and the expression levels of mRNA and its protein (Blancato *et al.,* 2004). In bladder carcinomas, this correlation is not yet clear, as the gene is also seen amplified in low-grade tumors (Schulz *et al.,* 1998). In cervical carcinoma, *c-MYC* amplification occurs in early stages and can lead to tumor progression (Abba *et al.,* 2004). In gastric carcinomas, its overexpression is a major factor that can be used to distinguish between well differentiated adenomas and adenocarcinomas. This alteration is present not only at the beginning of the disease, but seems to be related with its entire course (Kozma *et al.,* 2001).

c-ERBB-2, also known as *HER-2/neu*, is located in region 17q11-q12 (Yamamoto *et al.,* 1986) and is another oncogene that is often amplified in tumors of different origins, such as breast, ovary, bladder, stomach and lung (Takehana *et al.,* 2002; Lear-Kaul *et al.,* 2003); in some of them, it is a potential marker of prognosis. This gene encodes a transmembrane phosphoglucoprotein (p185) that resembles the epidermal growth factor



receptor (EGFR) which acts as a tyrosine kinase receptor, stimulating cell proliferation (Ohta *et al.,* 2001; Rabbani *et al.,* 2001; Krause *et al.,* 2000) (Figure 2).

In breast and lung tumors, *HER-2/neu* amplification induces overexpression of the protein in the cell membrane (Ohta *et al.,* 2001; Takehana *et al.,* 2002; Lear-Kaul *et al.,* 2003), which has been associated with a poor prognosis (Ohta *et al.,* 2001; Cianciulli *et al.,* 2003; Hirsch *et al.,* 2003). In gastric tumors, its overexpression is related to the presence of metastases (Nessling *et al.,* 1998; Vidgren *et al.,* 1999; Varis *et al.,* 2002) and to a development into the intestinal type (Becker *et al.,* 2000).

Amplification of the oncogene *HER-2/neu*, regardless of protein overexpression, has frequently been detected in bladder tumors, although it has not yet been correlated with the development, progression and clinical course of the disease (Adshead *et al.,* 1998; Brandau *et al.,* 2001; Ohta *et al.,* 2001). In these as well as in breast tumors, amplification and overexpression of the Her-2/neu protein have been investigated with the purpose of possibly directing the treatment of positive patients towards the use of Herceptin (trastuzumab), since the levels of gene amplification and protein expression are very similar to those found in patients with breast cancer (Ross & McKenna, 2001; Ross & Gray, 2003).

The drug Herceptin, a monoclonal antibody, works as a negative regulator of the protein Her-2/neu, inhibiting the signaling pathway of the cell cycle and blocking its progression. In vivo, trastuzumab inhibits angiogenesis and induces antibody-dependent cell cytotoxicity (Albanell *et al.,* 2003; Menard *et al.,* 2003). Besides Herceptin, other drugs are under study for use in patients with amplification of the oncogenes *c-MYC* and *c-MET* (Ma *et al.,* 2003). The protein c-myc takes part in several signaling cascades during the cell cycle and appears activated in tumor cells, promoting cell proliferation and regulating the expression of many target genes. Thus, inhibition of *c-MYC* expression could be sufficient to block tumor growth and to induce its regression (Hermeking *et al.,* 2003).



As mentioned before, activation of several oncogenes through gene amplification is related to the progression of several types of tumors. Many studies have shown that patients with gene amplification present a worse prognosis compared to those without amplification. We can therefore conclude that the amplification of these oncogenes plays an important role in the progression of carcinomas by increasing the risks for metastases and more aggressive tumors. So, they comprise valuable genetic markers and therapeutic targets for the development of effective drugs for the treatment of different types of tumors.

REFERENCES

Abba MC, Laguens RM, Dulout FN and Golijow CD (2004) The c-myc activation in cervical carcinomas and HPV 16 infections. Mut Reserch 557:151-158.

Adshead JM, Kessling AM and Ogden CW (1998) Genetic initiation, progression and prognostic markers in transitional cell carcinoma of the bladder: a summary of the structural and transcriptional changes, and the role of developmental genes. Br J Urol 82:503-512.

Albanell J, Codony J, Rovira A, Mellado B and Gascon P (2003) Mechanism of action of anti-HER2 monoclonal antibodies: Scientific update on trastuzumab and 2C4. Adv Exp Med Biol 532:253-268.

Bast Robert C, Kufe Donald W, Pollock Raphael E, Weichselbaum Ralph R and Holland James F (2000) Cancer Medicine. BC Decker Inc. 5th edition, Ontario.

Becker KF, Keller G and Hoefler H (2000) The use of molecular biology in diagnosis and prognosis of gastric cancer. Surg Oncol 9:5-11.

Blancato J, Singh B, Liu A, Liao DJ and Dickson RB (2004) Correlation of amplification and overexpression of the c-myc oncogene in high-grade breast cancer: FISH, in situ hybridization and immunohistochemical analyses. Br J Cancer 90:1612-1619.

Brandau S and Bohle A (2001) Bladder cancer. Molecular and Genetic Basis of Carcinogenesis. Eur Urol 39:491-497.

Cianciulli AM, Guadagni F, Benevolo M, Merola R, Giannarelli D, Marandino F, Vocaturo G, Mariani L and Mottelese M (2003) HER-2/neu oncogene amplification and chromosome 17 aneusomy in endometrial carcinoma: correlation with oncoprotein expression and conventional pathological parameters. J Exp Clin Cancer Res 22:265-271.



Cooper GM (2000) The cell: A molecular approach. 2nd edition. Sinauer Associates, Inc., Massachusetts.

Ethier SP (2003) Identifying and validating causal genetic alterations in human breast cancer. Breast Cancer Res Treat 78:285-287.

Ewen ME and Lamb J (2004) The activities of cyclin D that drive tumorigenesis. Trends in Mol Medicine 10:158-162.

Ghani-Abdel AS, Ghada EA, Naase M and Wells CA (2002) C-myc oncoprotein expression and gene amplification in apocrine metaplasia and apocrine change within sclerosing adenosis of the breast. The breast 11:466-472.

Hellman A, Zlotorynski E, Scherer SW, Cheung J, Vincent JB, Smith DI, Trakhtenbrot L and Kerem B (2002) A role for common fragile site induction in amplification of human oncogenes. Cancer Cell 1: 89-97.

Hermeking H (2003) The MYC oncogene as a cancer drug target. Curr Cancer Drug Targets 3: 163-175.

Herynk HM, Stoeltzing O, Reinmuth N, Parikh NU, Abounader R, Laterra J, Radinsky R, Ellis LM and Gallick GE (2003) Down-Regulation of c-Met Inhibits Growth in the Liver of Human Colorectal Carcinoma Cells. Cancer Res 63:2990-2996.

Hirsch FR, Scagliotti GV, Langer CJ, Varella-Garcia M and Franklin WA (2003) Epidermal growth factor family of receptors in preneoplasia and lung cancer: perspectives for target therapies. Lung Cancer 41:29-42.

Hastings PJ and Rosenberg SM (2002) In pursuit of a molecular mechanism for adaptive gene amplification. DNA Repair 1:111-123.

Huang X, Gollin SM, Raja S and Godfrey TE (2002) High-resolution mapping of the 11q13 amplicon and identification of a gene, TAOS1, that is amplified and overexpressed in oral cancer cells. PNAS 99:11369-11374.

Imoto I, Yang ZQ, Pimkhaokham A, Tsuda H, Shimada Y, Imamura M, Ohki M and Inazawa J (2001) Identification of cIAP1 as a candidate target gene within an amplicon at 11q22 in esophageal squamous cell carcinomas. Cancer Res 61:6629-6634.

Ishizuka T, Tanabe C, Sakamoto H, Aoyagi K, Maekawa M, Matsukura N, Tokunaga A, Tajiri T, Yoshida T, Terada M and Sasaki H (2002) Gene amplification profiling of esophageal squamous cell carcinomas by DNA array CGH. Biochem Biophys Res Commun 296:152-155.

Kozma L, Kiss I, Hajdu J, Szentkereszty Z, Szakall S and Ember I (2001) C-myc amplification and cluster analysis in human gastric carcinoma. Anticancer Res 21:707-710.

Krause FS, Feil G, Zumbragel A and Bichler KH (2000) Molecular genetic methods in the diagnosis of invasive bladder cancer. Anticancer Research 20:5015 – 5022.

Lear-Kaul KC, Yoon HR, Kleinschmidt-DeMasters BK, Mc Gavran L and Singh M (2003) Her-2/neu status in breast cancer metastases to the central nervous system. Arch Pathol Lab Med 127: 1451-1457.

Ma PC, Maulik G, Christensen J and Salgia R (2003) c-Met structure, functions and potential for therapeutic inhibition. Cancer Metastasis Rev 4:309-325.

Mai S and Mushinski FJ (2003) c-Myc Induced genomic instability. J Environm Pathol Toxicol and Oncol 3: 179-199.

Menard S, Pupa SM, Campiglio M and Tagliabue E (2003) Biologic and therapeutic role of HER-2 in cancer Oncogene 42:6570-6578.

Miller CT, Moy JR, Lin L, Schipper M, Normolle D, Brenner DE, Iannettoni MD, Orringer MB and Beer DG (2003) Gene amplification in esophageal adenocarcinomas and Barret´s with high-grade dysplasia. Clin Cancer Res 9:4819-4825.

Miyamoto R, Uzawa N, Nagaoka S, Hirata Y and Amagasa T (2003) Prognostic significance of Cyclin D1 amplification and overexpression in oral squamous cell carcinomas. Oral Oncol 39: 610-618.

Moreno-Bueno G, Rodriguez-Perales S, Sanchez-Estevez C, Hardisson D, Sarrio D, Prat J, Cigudosa JC, Matias-Guiu X and Palacios J (2003) Cyclin D1 gene (CCND1) mutations in endometrial cancer. Oncogene 22:6115-6118.

Nadal A and Cardesa A (2003) Molecular biology of laryngeal squamous cell carcinoma. Virchows Arch 442:1-7.

Nagasawa S, Onda M, Sasajima K, Makino H, Yamashita K, Takubo K and Miyashita M (2001) Cyclin D1 overexpression as a prognostic factor in patients with esophageal carcinoma. J Surg Oncol 78:208-214.

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 carcinomas. Carcinogenesis 23:19-24.

Nessling M, Solinas-Toldo S, Wilgenbus KK, Borchard F and Lichter P (1998) Mapping of chromosomal imbalances in gastric adenocarcinoma revealed amplified protooncogenes MYCN, MET, WNT2, and ERBB2. Genes Chromosomes Cancer 23:307-316.

Ohta JI, Miyoshi Y, Uemura H, Fujinami K, Mikata K, Hosaka M, Tokita Y and Kubota Y (2001) Fluorescence in situ hybridization evaluation of c-erbB-2 gene amplification and chromosomal anomalies in bladder cancer. Clin Cancer Res 7: 2463-2467.

Ormandy JO, Musgrove EA, Hui R, Daly RJ and Sutherland RL (2003) Cyclin D1, EMS1, and 11q13 amplification in breast cancer. Breast Cancer Research and Treatment 78:323-335.

Ozawa S, Kitagawa Y and Kitajima M (2002) Molecular alterations in esophageal cancer. Nippon Geka Gakkai Zasshi 103:457-462.



Rabbani F and Cordon-Cardo C (2000) Mutation of cell cycle regulators and their impact on superficial bladder cancer. Urol Clin North America 27: 83-102.

Robanus-Maandag EC, Bosch CA, Kristel PM, Hart AA, Faneyte IF, Nederlof PM, Peterse JL and van de Vijver MJ (2003) Association of C-MYC amplification with progression from the in situ to the invasive stage in C-MYC-amplified breast carcinomas. J Pathol 201:75-82.

Ross JS and Gray GS (2003) Target therapy for cancer: the HER-2/neu and Herceptin story. Clin Leadersh Manag Rev 17:333-340.

Ross JS, Fletcher JA, Bloom KJ, Linette GP, Stec J, Symmans WF, Pusztai L and Hortobagyi GN (2004) Targeted therapy in breast cancer: the HER-2/neu gene and protein. Mol Cell Proteomics 3: 379-398.

Saikawa Y, Kubota T, Otani Y, Kitajima M and Modbim Im (2001) Cyclin D1 antisense oligonucleotide inhibits cell growth stimulated by epidermal growth factor and induces apoptosis of gastric cancer cells. Jpn J Cancer Res 92:1102-1109.

Sarasin A (2003) An overview of the mechanisms of mutagenesis and carcinogenesis. Mut Research 544:99-106.

Schulz WA, Jankevicius F, Gerharz CD, Kushima M, van Roeyen CRC, Gobel P and Schimitz-Drager BJ (1998) Predictive value of molecular alterations of the prognosis of urothelial carcinoma. Cancer Detect Prevent 22:422-429.

Simon R, Struckmann K, Schraml P, Wagner U, Forster T, Moch H, Fijan A, Bruderer J, Wilber K, Mihatsch MJ, Gasser T and Sauter G (2002) Amplification pattern of 12q13-q15 genes (MDM2, CDK4, GLI) in urinary bladder cancer. Oncogene 21:2476-2483.

Tahara E (2004) Genetic pathways of two types of gastric cancer. IARC Sci Publ 157:27-349.

Takehana T, Kunitomo K, Kono K, Kitahara F, Iizuka H, Matsumoto Y and Fujino MA (2002) Status of c-erbB-2 in gastric adenocarcinoma: a comparative study of

immunohistochemistry, fluorescence in situ hybridization and enzyme-linked immunosorbent assay. Int J Cancer 98: 833-837.

Tsugawa K, Yonemura Y, Hirono Y, Fushida S, Kaji M, Miwa K, Miyazaki I and Yamamoto H (1998) Amplification of the c-met, c-erbB-2 and epidermal growth factor receptor gene in human gastric cancers: correlation to clinical features. Oncology 55: 475-481.

Tsujimoto H, Sugihara H, Hagiwara A and Hattori T (1997) Amplification of growth factor receptor genes and DNA ploidy pattern in the progression of gastric cancer. Virchows Arch 431: 383-389.

Varis A, Wolf M, Monni O, Vakkari ML, Kokkola A, Moskaluk C, Frierson H Jr, Powell SM, Knuutila S, Kallioniemi A and El-Rifai W (2002) Targets of gene amplification and overexpression at 17q in gastric cancer. Cancer Res 62:2625-2629.

Vidgren V, Varis A, Kokkola A, Monni O, Puolakkainen P, Nordling S, Forozan F, Kallioniemi A, Vakkari ML, Kivilaakso E and Knuutila S (1999) Concomitant gastrin and ERBB2 gene amplifications at 17q12-q21 in the intestinal type of gastric cancer. Genes Chromosomes Cancer 24:24-29.

Vielba R, Bilbao J, Ispizua A, Zabalza I, Alfaro J, Rezola R, Moreno E, Elorriaga J, Alonso I, Baroja A and de la Hoz C (2003) p53 and cyclin D1 as prognostic factors in squamous cell carcinoma of the larynx. Laryngoscope 113:167-172.

Watters AD, Latif Z, Forsyth A, Dunn I, Underwood MA, Grigor KM and Bartlett JM (2002) Genetic aberrations of c-myc and CCND1 in the development of invasive bladder cancer. Br J Cancer 87:654-658.

Yakut T, Egeli U and Gebitekin C (2003) Investigation of c-myc and p53 gene alterations in the tumor and surgical borderline tissues of NSCLC and effects on clinicopathologic behavior: by the FISH technique. Lung 181:245-258.

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Zajac-Kaye M (2001) Myc oncogene: a key component in cell cycle regulation and its implication for lung cancer. Lung Cancer 34:43-46.

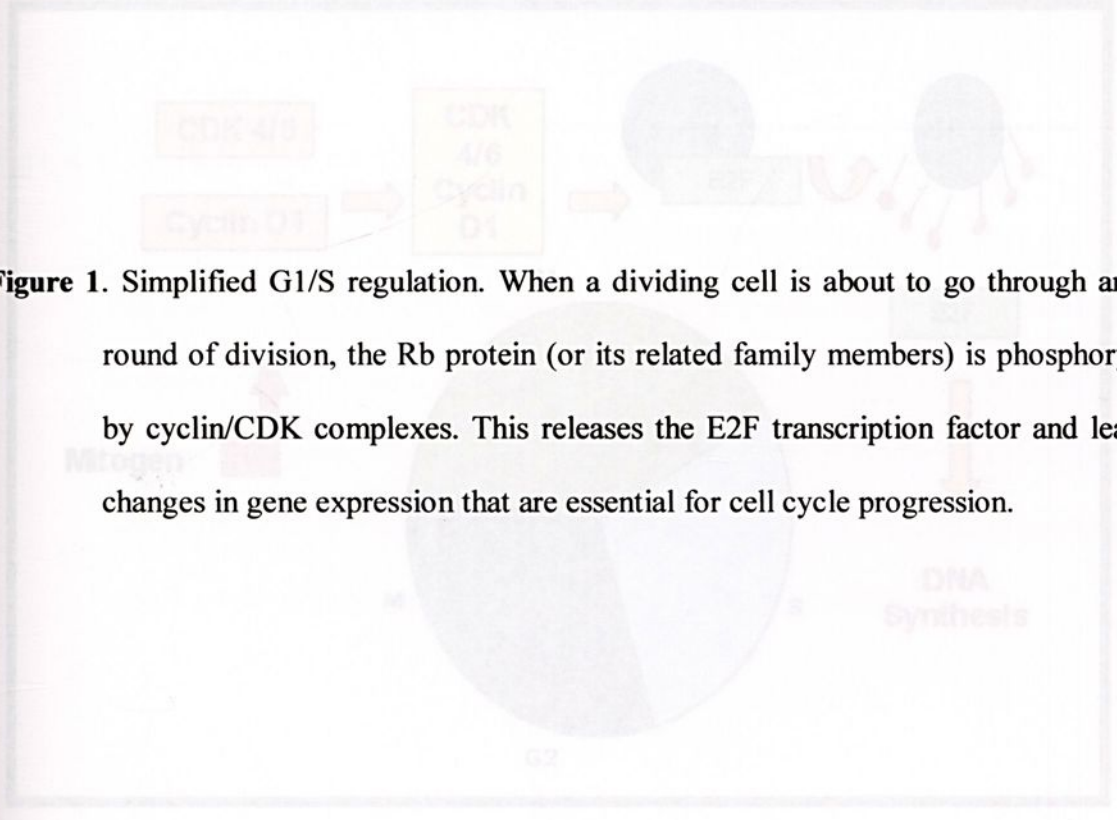


Figure 1. Simplified G1/S regulation. When a dividing cell is about to go through another round of division, the Rb protein (or its related family members) is phosphorylated by cyclin/CDK complexes. This releases the E2F transcription factor and leads to changes in gene expression that are essential for cell cycle progression.

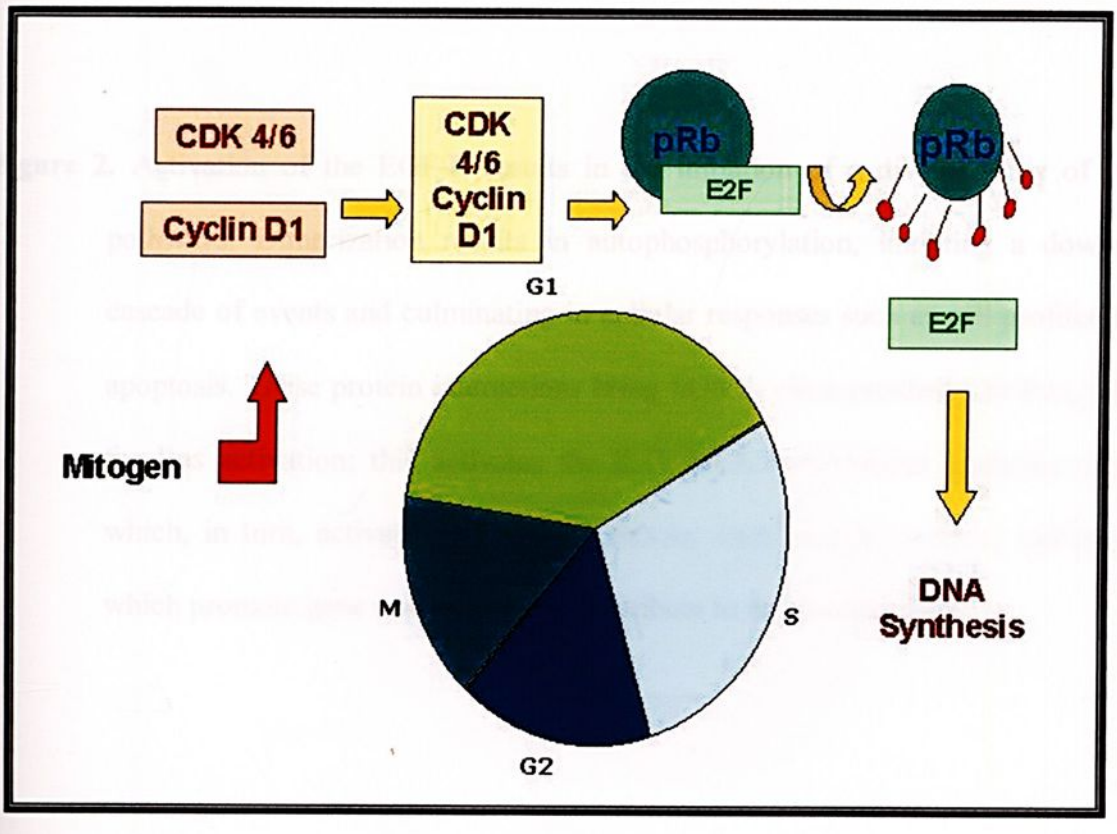
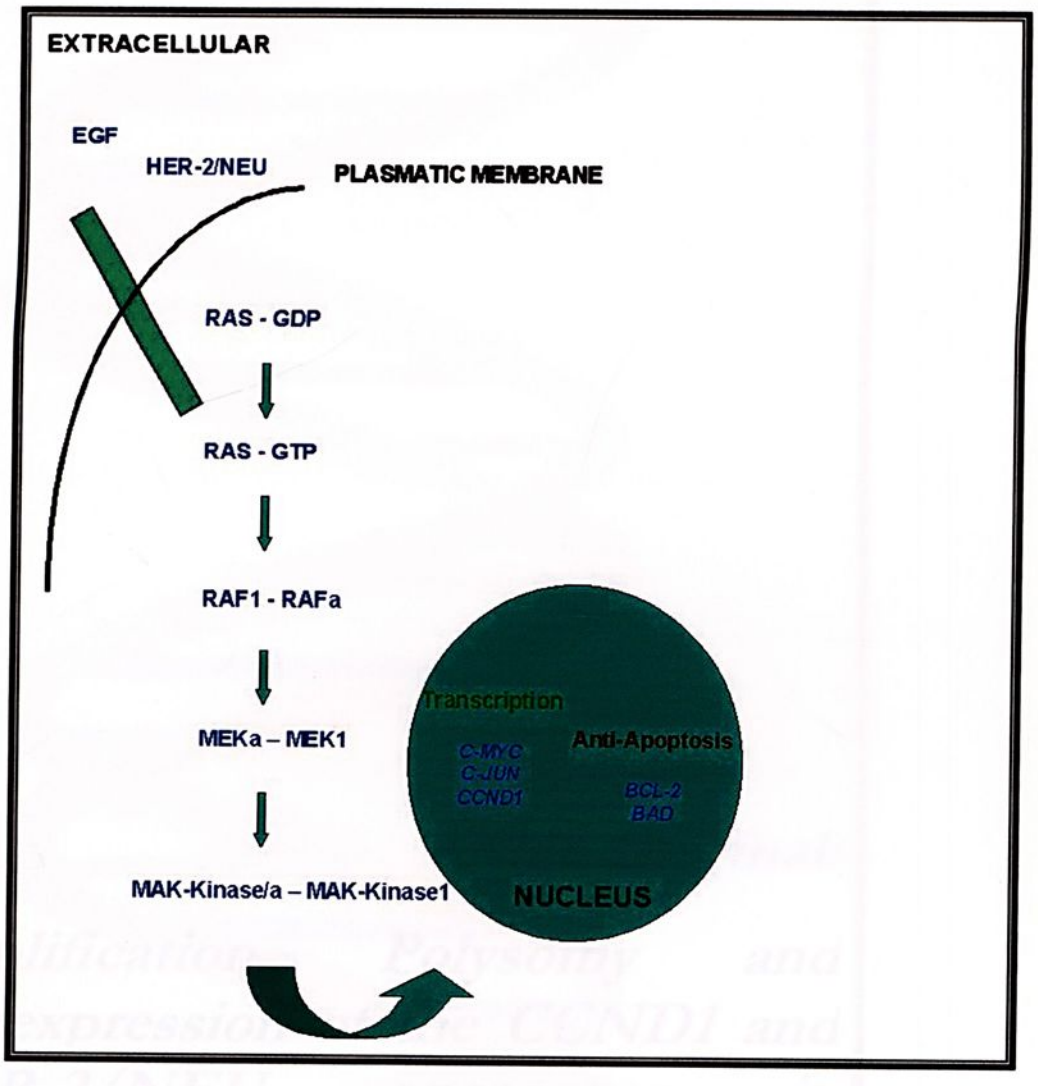

CDK 4/6
Cyclin D1
CDK 4/6 Cyclin D1
pRb
E2F
pRb
G1
E2F
Mitogen
M
S
G2
DNA Synthesis

Figure 2. Activation of the EGF-R results in the initiation of a diverse array of cellular pathways. Dimerization results in autophosphorylation, initiating a downstream cascade of events and culminating in cellular responses such as cell proliferation or apoptosis. These protein interactions bring SOS in close proximity to Ras, allowing for Ras activation; this activates the RAF-MEK-MAP-kinase signaling pathways which, in turn, activate transcription factors, such as c-fos, c-myc, and cyclin D, which promote gene expression and contribute to cell proliferation.


EXTRACELLULAR
EGF
HER-2/NEU
PLASMATIC MEMBRANE
RAS - GDP
RAS - GTP
RAF1 - RAFa
MEKa – MEK1
MAK-Kinase/a – MAK-Kinase1
Transcription
C-MYC
C-JUN
CCND1
Anti-Apoptosis
BCL-2
BAD
NUCLEUS

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Abstract

We made an evaluation of the contribution of amplification and high-balanced polysomy of both the *CCND1* and the *HER-2/neu* genes to the overexpression of proteins in esophageal and gastric cancers, and attempted to determine the association between these events and also with demographic and clinicopathological features. Regarding *CCND1*, gene copy gain by amplification (45%) was prevalent over polysomy (25%) in the cases of esophageal carcinoma, but in gastric adenocarcinoma the pattern observed was similar, i. e., 10% and 15%, respectively. However, for the *HER-2/neu* gene, polysomy was a more important mechanism than amplification in both esophageal (32.5% vs. 7.5%) and gastric cancers (15% vs 5%). Protein overexpression of Cyclin D1 was identified in 37.5% and 35%, and of Her-2/neu in 12.5% and 7.5% of the specimens of esophageal and gastric tumors, respectively. Kappa statistics revealed a fair agreement in both types of tumors only between overexpression and amplification of the *CCND1* gene, but not with polysomy. As for the *HER-2/neu* gene, neither amplification nor polysomy showed an association with protein overexpression in esophageal carcinoma, but in gastric adenocarcinoma a fair agreement was observed between amplification and polysomy and the level of protein expression. This finding emphasizes that high-balanced polysomy could contribute to a high Her-2/neu protein level, at least in gastric adenocarcinoma. Our data showed no association between the *CCND1* and *HER-2/neu* genes and protein status with demographic and clinicopathological features, except for an association with alcohol consumption and the *CCND1* gene or protein levels, in both esophageal and gastric cancers.

1. Introduction

Cancers of the gastrointestinal tract, including the esophagus and stomach, show increasing rates of mortality and morbidity in the world [1,2]. The interaction of both environmental and genetic factors contributes to the etiology and pathogenesis of these aggressive cancers [2,3,4]. Smoking and alcohol consumption are appointed as major environmental agents in esophageal and gastric carcinogenesis [5,6], besides dietary habits and bacterial infection by *Helicobacter pylori* in gastric cancer [1,7].

Recent molecular advances have made it clear that the uncontrolled proliferation of carcinoma cells involves an accumulation of oncogene and tumor-suppressor gene abnormalities [8], which control the cell cycle. Among the genetic alterations involved, the gain of a number of gene copies by amplification and/or polysomy appears to be an important mechanism in carcinogenesis and tumoral progression [9-11]. Amplification of the *CCND1* and/or *HER-2/neu* genes has been demonstrated in several human tumor types, such as breast [12-14], bladder [14], squamous cell carcinoma of the head and neck [15,16], esophageal [9,17] and gastric cancers [18,19], and is thought to be an important determinant of the biologic behavior of carcinomas [17, 19-21].

Additionally, both genes are involved in the activation of cell proliferation by different pathways. The *CCND1* gene (*PRAD1, Cyclin D1*), localized at chromosomal band 11q13 [22], encodes the protein Cyclin D1, a member of the cyclin family, involved in the G1/S transition. Its alteration can cause deregulation of the cell cycle, resulting in oncogenesis [23,24]. The gene *HER-2/neu* (*ERBB2*), localized in chromosome region 17q11.2-q12, encodes a transmembrane glycoprotein that is a member of the epidermal growth factor receptor family [25]. The protein Her-2/Neu or p185 acts as a tyrosine kinase

receptor, stimulating cell proliferation [26,27] and therefore being potentially involved in the growth and progression of malignant cells [21].

Immunohistochemical assays have demonstrated overexpression of the Cyclin D1 and Her-2/neu proteins in some types of cancers, mainly breast [28], osteosarcomas [21], urothelial carcinoma [29,30], esophageal carcinoma [10,19,21,31,32] and gastric cancer [33-35]. The status of these proteins has been related to clinicophatological factors, so they can be potential biomarkers for cancer prognosis.

There is so far a limited number of studies that evaluated simultaneously the amplification and overexpression of the *CCND1* and *HER-2/neu* genes in esophageal [10, 19] and gastric carcinomas [18]. Moreover, an exact relation between overexpression and gene amplification has not been observed [10,21,36], nor is there a precise correlation of these alterations with prognosis and survival [21,36].

Although gene amplification appears to be the main mechanism leading to overexpression of both Cyclin D1 and Her-2/neu proteins in carcinogenesis, the gain of a number of gene copies by high-polysomy may also be an important mechanism in those tumors, with positive protein immunostaining but no amplification, as recently reported in breast cancer [37].

The purpose of the present study was to evaluate the relationship of amplification and polisomy of the genes *CCND1* and *HER-2/*neu with overexpression of proteins in patients with esophageal carcinoma and gastric adenocarcinoma. Thus, we evaluated the gene status (amplification and polysomy) and overexpression of proteins, and determined the agreement between these events, as well as the association of gene status and overexpression with demographic and clinicopathological features.

2. Material and methods

2.1. Patients

A total of 80 specimens (fresh tumor tissue and paraffin-embedded specimens) were obtained from 40 patients with esophageal squamous cell carcinoma (34 males and 6 females) and 40 patients with gastric adenocarcinoma (30 males and 10 females), before any chemotherapy and radiotherapy. All patients were diagnosed and operated on at the Department of Surgery of Hospital de Base, in São José do Rio Preto, SP, Brazil. The mean age of the patients with esophageal carcinoma was 63.4 years (range 46-96 years), and the mean age of the patients with gastric adenocarcinoma was 62.3 years (range 28-93 years). The esophageal tumors were graded according to the World Health Organization: 1 tumor was classified as well differentiated, 30 as moderately differentiated, 4 as poorly differentiated, and 5 as undifferentiated. Gastric adenocarcinoma was classified by characteristic histological features into diffuse (25 specimens) or intestinal (15 specimens) types [38]. Biopsies obtained from the same patients, but removed from distant surgery areas, were used to establish the cut-off level for the gene status (12 normal esophageal mucosae and 14 normal gastric mucosae) and protein status (6 normal esophageal mucosae and 7 normal gastric mucosae).

Written informed consent was obtained from all patients, and the project was approved by the National Research Ethics Committee.

2.2. Fluorescence *in situ* hybridization (FISH)

FISH assays were performed on cell suspensions using a Cyclin D1 probe kit (Vysis), including the LSI Cyclin D1 sequence labeled in Spectrum Orange, with the chromosome 11 centromere sequence labeled in Spectrum Green, and the HER-2/neu kit

(Q-bioGene), including the probe HER-2/neu sequence, labeled with Rhodamine, with the chromosome 17 centromere labeled with fluorescein, following the instructions given by the manufacturer. All specimens were mechanically and enzymatically disaggregated with 0.008% pepsin, and the single-cell suspensions obtained were treated with hypotonic solution (0.075M KCl) and fixed in methanol-acetic acid (3:1) overnight. After washes in 60% acetic acid, the resulting cell suspension was dropped onto microscope slides. Briefly, the slides were treated with 70% acetic acid at room temperature for 5 min. Then, a solution containing the Cyclin D1 or HER-2/neu probes was applied to the specimens and co-denatured at 80°C for 5-8 min. Afterwards, the slides were hybridized overnight (16 to 18 hours) at 37°C in a moist chamber. After the post-hybridization washes, the slides were counterstained with DAPI (4',6-diamino-2-phenylindole).

The slides were analyzed under an Olympus BX60 microscope. The number of signals corresponding to chromosome 11 centromeres and to the *CCND1* gene were scored together, and the number of chromosome 17 centromere signals was scored together with those corresponding to the *HER-2/neu* gene in about 250 nuclei, in accordance with the criteria described by Eastmond et al. [39]. The gene-to-centromere (G/C) ratio was calculated for each case, that is, the total number of gene signals observed was divided by the total number of centromere signals. For each FISH probe tested, the status of the chromosome used as a control was classified as either disomy or polysomy, when on average 2 or 3 or more copies, respectively, were scored per nucleus. Gene gain was considered either by amplification or by high-balanced polysomy, recognized as overrepresentation [10]. Gene amplification assessed by multiple copies of the gene in any chromosomal status was classified as high-level whenever the gene/centromere ratio was >

2.0 or with clustered copies (groups of many copies of the gene), while high-balanced polysomy was considered as positive only in the cases with high frequencies (>15%).

2.3. Immunohistochemistry (IHC)

Tumor material consisted of paraffin-embedded specimens fixed in 10% formalin. Sections were cut at 5μm and dried for 1 h at 65°C. After deparaffinization and rehydration of the sections, antigen retrieval was performed in a Tris-EDTA solution pH 9.0 for Cyclin D1, and 10mM citrate pH 6.0 for Her-2/neu antibody. All further steps were performed using an automated system (Cadenza). Endogenous peroxidases were inhibited by 20 min incubation in 3% H_2O_2. Then, the sections were incubated overnight at room temperature with the monoclonal Cyclin D1 or Her-2/neu antibodies (Dako), used at a dilution of 1:100 and 1:200, respectively. Immunodetection was achieved using the NOVOSTATIN Super ABC kit (NOVOCASTRA), for 35 min at room temperature. The sections were then incubated with diaminobenzidine for 5 min and counterstained with hematoxylin for 15 sec. Amigdala and breast carcinomas were used as positive controls for Cyclin D1 and Her-2/neu antibodies, respectively.

All specimens were revised by a single pathologist and only the IHC status was evaluated. For Cyclin D1 immunostaining about 500 nuclei were counted. The index of positivity was defined as a value greater than 30% and 20% of nuclei showing strong Cyclin D1 immunostaining, in esophageal carcinomas and gastric adenocarcinomas, respectively, according to the mean frequencies observed in the respective normal mucosae. The membrane staining intensity and pattern considered for scoring were in accordance with the criteria approved by the FDA (Food and Drug Administration) for the HER-2/neu

antibody: 0, no staining or membrane staining, observed in less than 10% of tumor cells; 1+, partial membrane staining, in more than 10% of tumor cells and membrane, not circumferential, staining; 2+, circumferential, weak to moderate staining, observed in more than 10% of tumor cells; and 3+, strong circumferential membrane staining, observed in more than 10% of tumor cells. Scores 2+ and 3+ were considered positive. Cytoplasmatic immunostaining was not considered for either protein. Areas that were poorly preserved, crushed, cauterized, folded, or retracted were specifically avoided.

2.4. Statistics

FISH and IHC results were compared with demographic and clinicopathological information by Fisher's exact test, using the GraphPad Instat computer software program. The level of significance was set at $P<0.05$. A *Kappa* statistical test was made to examine the agreement between gene and protein status for the two molecular targets, using the website http://www.lee.dante.br/pesquisa/kappa/index/html [40].

3. Results

Cyclin D1 and HER-2/neu amplification, polysomy and overexpression

Tables 1 and 2 summarize the results concerning gene status (amplification and polysomy) and overexpression of the *CCND1* and *HER-2/neu* genes in normal and tumoral tissues of the esophagus and stomach. None of the samples from either the esophagus or stomach that had a normal histology displayed gene gain or appreciable immunostaining for Cyclin D1 and Her-2/neu proteins, thus none of them was defined as positive.

High levels of amplification (G/C>2.0 or cluster) or high-balanced polysomy (>15%) of the *CCND1* gene were identified in a total of 28 out of 40 (70%) of the esophageal carcinomas and 10 out of 40 (25%) of the gastric adenocarcinomas (Table 1). Esophageal carcinoma showed higher levels of gene amplification than of polysomy [18/40 (45%) vs 10/40 (25%)], while gastric adenocarcinoma showed similar patterns [4/40 (10%) vs 6/40 (15%)]. *HER-2/neu* amplification or polysomy was found in a total of 16/40 (40%) of esophageal carcinomas and 8/40 (20%) of the gastric adenocarcinomas (Table 1). Balanced polysomy was more prevalent than amplification in both esophageal [13/40 (32.5%) vs. 3/40 (7.5%)] and gastric cancers [6/40 (15%) vs. 2/40 (5%)]. Interestingly, in both types of tumors, most specimens did not show simultaneously cluster and high-balanced polysomy, except for a single case of esophageal and another one of gastric cancer regarding the *HER-2/neu* gene. Figure 1 illustrates the gene gain (amplification and polysomy) of the *CCND1* and *HER-2/neu* genes in esophageal and gastric cancers.

The Cyclin D1 protein was found to be overexpressed with positive immunostaining in over 30% of the nuclei (range 30.8 to 72.8%) in esophageal carcinoma and in over 20% (range 20.2 to 69.3%) in gastric adenocarcinoma, in 15/40 (37.5%) and 14/40 (35%) of the tumors, respectively (Table 2). High-level overexpression of the Her-2/neu protein (scores 2+ and 3+) was seen in 5/40 (12.5%) and 3/40 (7.5%) of the esophageal carcinomas and gastric adenocarcinomas, respectively (Table 2). Figure 2 illustrates the Cyclin D1 and Her-2/neu overexpression in esophageal and gastric cancers.

Relationship between amplification and polysomy with overexpression of* Cyclin D1 *and* Her-2/neu *proteins

According to *Kappa* statistics (Table 3), in both esophageal and gastric tumors, the combination of gene status (amplification and polysomy) with protein expression of the *CCND1* gene showed only a fair agreement between amplification and overexpression (*Kappa*=0.333 and 0.340, respectively). Moreover, only a single specimen of esophageal carcinoma and 7 of gastric adenocarcinoma had an overexpressed Cyclin D1 protein without displaying either amplification or polysomy.

Regarding *HER-2/neu*, neither amplification nor polysomy showed an agreement with protein overexpression in the esophageal carcinomas (*Kappa=0.172* and *0.186*, respectively), but in gastric adenocarcinomas a fair agreement was observed between amplification and polysomy and the protein expression level (K*appa=0.360 and 0.383*, respectively). Conversely, a single specimen of esophageal and none of gastric cancer was positively immunostained, but without amplification or polysomy.

Combined analyses of Cyclin D1 and HER-2/neu for gene status and overexpression

The combined analyses of the two molecular targets *CCND1* and *HER-2/neu* in each tumor (Table 4) revealed that 12/40 (30%) of esophageal carcinoma and only 3/40 (7.5%) of gastric cancers showed simultaneously gene gain by amplification and/or polysomy for both genes. Thus, no agreement was found regarding this parameter between esophageal and gastric tumors (*Kappa=0.030* and *0.100*, respectively). Similarly, no agreement was detected between Cyclin D1 and Her-2/neu protein overexpression in the specimens of both esophageal and gastric cancers (*Kappa=0.010* and *-0.001*, respectively),



since only 2/40 (5%) of esophageal carcinomas and 1/40 (2.5%) of gastric cancers co-overexpressed both proteins.

Relationship between gene status (amplification and polysomy) and overexpression of CCND1 and HER-2/neu with demographic and clinicopathological features

The evaluation of an association between *CCND1* and *HER-2/neu* gene gain by amplification or polysomy and protein overexpression with demographic and clinicopathological characteristics (Tables 5 and 6) revealed a statistically significant association only between *CCND1* gene gain (amplification and polysomy) and alcohol consumption in gastric adenocarcinoma (*P=0.0260* and *0.0295*, respectively). Cyclin D1 overexpression in esophageal carcinoma also showed a significant association with alcohol consumption (*P=0.0175*). In the other comparisons (age, gender, smoking, survival, histological grade and *H. pylori* infection) no significant differences were found for either gene and protein status.

4. Discussion

In the current study, both the *CCND1* and the *HER-2/neu* molecules were evaluated for gene status by amplification and polysomy and protein overexpression levels, in a series of cases of esophageal carcinoma and adenocarcinoma of the stomach. The number of copies per cell of each gene and centromere of interest was correlated with a protein score. Amplifications of these genes as well as overexpression of the corresponding proteins appear to play an important role in esophageal [8,10,17,21] and gastric carcinogenesis [18,21].

To our best knowledge, this is the first study that carried out a simultaneous evaluation of *CCND1* and *HER-2/neu* gene and protein status in esophageal and gastric cancer. Of the two genes evaluated, *CCND1* was the more frequently amplified and overexpressed one in both tumors, while for the *Her-2/neu* gene the gain of copies occurred mainly by high-balanced polysomy, that must have contributed to an increased protein expression, leading to positive immunostaining in some of the cases without amplification.

In esophageal carcinomas, our results showed that gene amplification (45% of cases) was the main mechanism responsible for overexpression of the Cyclin D1 protein, as compared to polysomy (25% of cases). These findings reinforce the evidence that *CCND1* gene amplification is associated with esophageal carcinogenesis, with frequencies ranging from 20% to 80% of the cases [10,15,17,41]. However, in gastric cancer, the *CCND1* gene was commonly not amplified, and polysomy was not frequent either, both having been observed in only 10-15% of the cases. This finding is in accordance with other studies that reported frequencies of approximately 16.6% of *CCND1* amplification [35], therefore not appearing to play a significant role in the progression this type of cancer [42,43].

Additionally, in this study, the Cyclin D1 protein was commonly overexpressed in both esophageal and gastric carcinomas (37.5% and 35%, respectively), and its incidence rate falls within the wide range of 26 to 71% of esophageal carcinoma [10,31,32] and 25 to 50% of gastric cancer patients reported previously [24,31,33,35]. Gao et al. [35] suggested that positive expression of the Cyclin D1 protein may be an early event in gastric carcinoma pathogenesis, tending to remain stable throughout the progression of infiltration and metastasis. Thus, in a proportion of gastric cancer cases, the increased protein production must occur by another mechanism beyond amplification, as for example

high polysomy, observed in 17.5% of cases by Gao et al. [35] and in 15% in the current study.

When we evaluated the agreement of the cases with amplification or polysomy which simultaneously overexpressed the protein, we observed a fair agreement only between amplification and overexpression of the *CCND1* gene, and rather in esophageal carcinoma (25% of cases) than in gastric adenocarcinoma (10%).

On the other hand, while in oral squamous cell carcinomas Myiamoto et al. [15] found 100% of cases with amplification and overexpression of *CCND1*, other studies have reported an association in 30-70% of esophageal carcinoma cases with *CCND1* amplification and overexpression [10,41,45]. In gastric cancer, this association is lower, that is, approximately 17% [35].

Additionally, the Cyclin D1 protein was not expressed in all cases with amplification or polysomy, in both esophageal (14 cases) and gastric cancers (3 cases). Conversely, only a single case of esophageal carcinoma and 7 cases of gastric adenocarcinoma displayed overexpression without gene gain. This situation has been described in esophageal carcinoma [10,31], colon adenocarcinoma [31,46], breast cancer [47,48] and gastric carcinoma [35]. Probably other pathways allow Cyclin D1 expression in addition to amplification, such as alterations in the synthesis or stability of the Cyclin D1 protein [49], alterations in the *CCND1* promoter or abnormal regulation by transcription factors, which could both upregulate the expression of mRNA and induce the production of more Cyclin D1 protein [35]. Moreover, Gao et al. [35] observed that the expression of the Cyclin D1 protein was affected more directly by the mRNA level than by the gene amplification.



Interestingly, the evaluation of the *HER-2/neu* gene status revealed that high-polysomy was the prevalent pattern regarding the gain of *HER-2/neu* gene copies, in both esophageal (32.5%) and gastric cancer (15%) cases, as compared to gene amplification, observed only in 7.5% and 5.0% of cases, respectively. Similarly, Sunpaweravong et al. [10] reported a low level of amplification (2%) in comparison to gene overrepresentation by balanced polysomy (31%) in esophageal carcinoma. In gastric adenocarcinoma, Ishikawa et al. [50] also reported low frequencies of *HER-2/neu* amplification.

Polysomies 11 and 17 could have an additive effect with *CCND1* or *HER-2/neu* amplification and contribute to an overall increase of the number of gene copies in the tumor cells. In breast cancer, Lal et al. [37] showed the impact of polysomy 17 on Her-2/neu protein overexpression in the cases without gene amplification, evidencing polysomy 17 as a contributing factor to strong positive protein immunostaining. In squamous cell carcinoma of the pharynx and larynx, the polysomies 11 and 17 were found in 60 and 72% of cases, respectively [51], while in gastric adenocarcinoma Gobbo-César et al [52] displayed high-polisomy of chromosomes 3, 7, 8, 9 and 17. High-polysomy is a chromosome aberration that in many cases suggests a global genetic instability of the tumor, affecting more than one chromosome, and correlates with the proposed models of tumor progression in which this progression is the result of a series of sequential changes in the DNA content of the tumor [11].

In the present study, the expression of the Her-2/neu protein showed a lower incidence than that of the Cyclin D1 protein, in both esophageal and gastric carcinomas (12.5% and 7.5%, respectively), but in accordance with the 15 to 56% incidence reported for esophageal carcinoma [21,36,53] and with the wide range (8.0 to 53%) found in gastric cancer (18,20,37,54].



The evaluation of Her-2/neu protein and gene levels in the same specimens showed no agreement in esophageal carcinoma, but revealed a fair agreement in gastric cancer, supporting the hypothesis that a gain in copy number is not a critical mechanism for protein overexpression. For the *HER-2/neu* gene, this dissociation has been suggested to occur due to point mutation, translocation, transcriptional upregulation, heterodimerization with *HER-2* (cross-talk with *HER-2*), increased co-expression of ligands and alterations in molecules that control receptor signaling output, leading to protein overexpression [30, 55].

We have shown that *CCND1* and *HER-2/neu* are not associated with each other, whether at the gene or the protein levels, in both esophageal and gastric cancer. These results are concordant with those presented by Sunpaweravong et al. [10], who did not find an association between *CCND1, EGFR* and *HER-2/neu* in esophageal carcinoma either. Thus, there is no evidence of inter-gene interaction (*CCND1* and *HER-2/neu*) in the carcinogenesis process of the esophagus and stomach. The amplification and overexpression of *CCND1* and *HER-2/neu* in distinct tumors suggests alternative activation mechanisms of the tumorigenesis pathway [10].

Despite its low level of amplification and overexpression in patients with esophageal and gastric tumors, *HER-2/neu* appears to be an interesting molecular marker for targeted therapy. Takehana et al. [18] suggested that the mechanisms of overexpression and amplification of *HER-2/neu* in gastric adenocarcinoma seem to be similar to those well-established in breast cancers. In the OE19 esophageal adenocarcinoma cell line, Dalbergh et al. [19] observed growth inhibition after incubation with trastuzumab. So, patients with esophageal and gastric adenocarcinoma with Her-2/neu amplification or overexpression are potential candidates for an adjuvant therapy using humanized monoclonal antibody [18,19,54].



The relationship between *CCND1* and *HER-2/neu* amplification or overexpression and clinicopathological parameters in esophageal and gastric cancers has been controversial. In agreement with previous studies [18,20,24,32,35], our data show no association between *CCND1* and *HER-2/neu* amplification or overexpression with demographic and clinicopathological markers, including patient gender, age, smoking, histological grade, *H. pylori* infection and survival, in both esophageal and gastric cancer. However, our study revealed an association only of alcohol consumption with *CCND1* amplification and polysomy in gastric cancer patients, and with Cyclin D1 overexpression in patients with esophageal carcinoma, evidencing a gene-environment interaction. Alcohol consumption is an environmental factor that plays an important role in the carcinogenesis of the esophagus and stomach [6], because N-nitroso compounds are directly linked to carcinogenesis [43, 56]. However, further studies are needed to clarify the correlation between these genes and clinicopathological features.

In conclusion, our results indicate that the *CCND1* gene is more closely involved in the carcinogenesis of the esophagus and stomach than *HER-2/neu*, mainly due to amplification. Interestingly, at the level that could be demonstrated by IHC staining in some specimens of esophageal and gastric tumors, high-balanced polysomy of the *HER-2/neu* gene in addition to amplification was an important mechanism leading to increased Her-2/neu protein expression. Thus, it must be an event to be considered separately from amplification in the studies on gene status evaluation. Although an exact association between gene status and protein levels was not seen, probably as a consequence of the fact that some specimens showed amplification or polysomy without protein overexpression, the IHC assay mainly of the Cyclin D1 protein appears to be a suitable method to be employed as a biological marker in studies on esophageal and gastric carcinogenesis.



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5. References

[1] Hohenberger P, Gretschel S. Gastric cancer. Lancet 2003; 362(9380):305-315. Review.

[2] Lehrbach DM, Nita ME, Cecconello I. Molecular aspects of esophageal squamous cell carcinoma carcinogenesis. Arq Gastroenterol 2003; 40(4):256-261. Epub 2004 May 31. Review.

[3] Werner M, Becker KF, Keller G, Hofler H. Gastric adenocarcinoma: pathomorphology and molecular pathology. J Cancer Res Clin Oncol 2001; 127(4):207-216. Review.

[4] Heavey PM, Rowland IR. Microbial-gut interactions in health and disease. Gastrointestinal cancer. Best Pract Res Clin Gastroenterol. 2004; 18(2):323-336. Review.

[5] World Cancer Research Fund. Food, nutrition and the prevention of cancer: a global perspective. Washington, DC: World Cancer Research Fund, 1997.

[6] Wünsch-Filho V. The epidemiology of oral and pharynx cancer in Brazil. Oral Oncology 2002; 38:737-746.

[7] Tahara E. Genetic pathways of two types of gastric cancer. IARC Sci Publ 2004; (157):327-349. Review.

[8] Kagawa Y, Yoshida D, Hirai T, Toge T. Significance of the expression of $p27^{Kip1}$ in esophageal squamous cell carcinomas. Dis Esophagus 2000; 13(3):179-184.

[9] Shiomi H, Sugihara H, Kamitani S, Tokugawa T, Tsubosa Y, Okada K, Tamura H, tam T, Kodama M, Hattori T. Cytogenetic heterogeneity and progression of esophageal squamous cell carcinoma. Cancer Genet Cytogenet 2003; 147(1):50-61.

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

[11] Ribal MJ, Alcaraz A, Mengual L, Carrio A, Lopez-Guillermo A, Mallofre C, Palou J, Gelabert A, Villavicencio H. Chromosomal high-polysomies predict tumour progression in T1 transitional cell carcinoma of the bladder. Eur Urol 2004; 45(5):593-599.

[12] Simpson JF, Quan DE, O'Malley F, Odom-Mayon T, Clarke PE. Amplification of CCND1 and expression of its protein product, cyclin D1, in ductal carcinoma in situ of the breast. Am J Pathol 1997;151:161-168.

[13] Ormandy CJ, Musgrove EA, Hui R, Daly RJ, Sutherland RL. Cyclin D1, EMS and 11q13 amplification in breast cancer. Breast Cancer Res Treat. 2003; 78(3):323-335.

cm 1 2 3 4 5 6 7 unesp 11 12 13 14 15 16 17 18

[14] Ewen ME, Lamb J. The activities of cyclin D1 that drive tumorigenesis. Trends Mol Med 2004; 10(4):158-162.

[15] Myiamoto R, Uzawa N, Nagaoka S, Hirata Y, Amagasa T. Prognostic significance of cyclin D1 amplification and overexpression in oral squamous cell carcinomas. Oral Oncol 2003; 39(6):610-618.

[16] Niméus E, Baldetorp B, Bendahl P, Rennstam K, Wennerberg J, Akervall J, Ferno M. Amplification of the cyclin D1 gene is associated with tumour subsite, DNA non-diploidy and high S-phase fraction in squamous cell carcinoma of the head and neck. Oral Oncol 2004; 40(6):624-629.

[17] Manoel-Caetano FS, Borim AA, Caetano A, Cury PM, Silva AE. Cytogenetic alterations in chagasic achalasia compared to esophageal carcinoma. Cancer Genet Cytogenet 2004; 149:17-22.

[18] Takehana T, Kunitomon K, Kono K, Kitahara F, Iizuka H, Matsumoto Y, Fujino MA, Ooi A. Status of c-erbB-2 in gastric adenocarcinoma: a comparative study of immunohistochemistry, fluorescence in situ hybridization and enzyme-linked immuno-sorbent assay. Int J Cancer 2002; 20;98(6):833-837.

[19] Dahlberg PS, Jacobson BA, Dahal G, Fink JM, Kratzke RA, Maddaus MA, Ferrin LJ. ERBB2 amplifications in esophageal adenocarcinoma. Ann Thorac Surg 2004; 78(5):1790-800; discussion 1790-800.

[20] Dursun A, Poyraz A, Celik B, Akyol G. Expression of c-erbB-2 oncoprotein in gastric carcinoma: correlation with histopathologic characteristics and analysis of Ki-67. Pathol Oncol Res 1999; 5(2):104-106.

cm 1 2 3 4 5 6 7 unesp 11 12 13 14 15 16 17 18

[21] Akamatsu M, Matsumoto T, Oka K, Yamasaki S, Sonque H, Kajiyama Y, Tsurumaru M, Sasai K. c-erbb-2 oncoprotein expression related to chemoradioresistance in esophageal squamous cell carcinoma. Int J Radiat Oncol Biol Phys 2003; 57(5):1323-1327.

[22] Motokura T, Arnold A. Cyclin D1 and oncogenesis. Current Opin Genet Devel 1993; 3:5-10.

[23] Ikeguchi M, Sakatani T, Ueta T, Kaibara N. Cyclin D1 expression and retinoblastoma gene protein (pRB) expression in esophageal squamous cell carcinoma. J Cancer Res Clin Oncol 2001; 127:531-536.

[24] Ahn MJ, Kim BH, Jang SJ, Hong EK, Lee WM, Baik HK, Park HK, Lee CB, Ki M. Expression of cyclin D1 and cyclin E in human gastric carcinoma and its clinicopathologic significance. J Korean Med Sci 1998;13(5):513-518.

[25] Yamamoto T, Ikawa S, Akiyama T. Similarity of protein encoded by the human c-erbB-2 gene to epidermal growth factor receptor. 1986; 319:230-234.

[26] Ohta JI, Miyoshi Y, Uemura H, Fujinami K, Mikata K, Hosaka M, Tokita Y, Kubota Y. Fluorescence in situ hybridization evaluation of c-erbB-2 gene amplification and chromosomal anomalies in bladder cancer. Clin Cancer Res 2001;7(8):2463-2467.

[27] Rabbani F, Cordon-Cardo C. Mutation of cell cycle regulators and their impact on superficial bladder cancer. Urol Clin North Am 2000;27:83-102.

[28] Vera-Roman JM, Rubio-Martinez LA. Comparative assays for the HER-2/neu oncogene status in breast cancer. Arch Pathol Lab Med 2004; 128(6):627-633.

[29] Coogan CL, Estrada CR, Kapur S, Bloom KJ. HER-2/neu protein overexpression and gene amplification in human transitional cell carcinoma of the bladder. Urology 2004; 63(4):786-790.

[30] Pinieux G, Colin D, Vincent-Salomon A, Couturier J, Amsellem-Ouazana D, Beuzeboc P, Vieillefond A. Confrontation of immunohistochemistry and fluorescent in situ hybridization for the assessment of HER-2/neu (c-erbb-2) status in urothelial carcinoma. Virchows Arch 2004; 444(5): 415-419. Epub 2004 Mar 17.

[31] Arber N, Gammon MD, Hibshoosh H, Britton JA, Zhang Y, Schonberg JB, Roterdam H, Fabian I, Holt PR, Weinstein B. Overexpression of Cyclin D1 occurs in both squamous carcinomas and adenocarcinomas of the esophagus and in adenocarcinomas of the stomach. Hum Pathol 1999; 30(9):1087-92.

[32] Nagasawa S, Onda M, Sasajima K, Makino H, Yamashita K, Takubo K, Miyashita M. Cyclin D1 overexpression as a prognostic factor in patients with esophageal carcinoma. J Surg Oncol 2001; 78(3):208-214.

[33] Aoyagi K, Koufuji K, Yano S, Murakami N, Terasaki Y, Yamasaki Y, Takeda J, Tanaka M, Shirouzu K. Immunohistochemical study on the expression of cyclin D1 and E in gastric cancer. Kurume Med J 2000; 47(3):199-203.

[34] Feakins RM, Nickols CD, Bidd H, Walton SJ. Abnormal expression of pRb, p16, and cyclin D1 in gastric adenocarcinoma and its lymph node metastases: relationship with pathological features and survival. Hum Pathol 2003; 34(12):1276-1282.



[35] Gao P, Zhou GY, Liu Y, Li JS, Zhen JH, Yuan YP. Alteration of cyclin D1 in gastric carcinoma and its clinicopathologic significance. World J Gastroenterol. 2004; 15;10(20):2936-2939.

[36] Hardwick RH, Barham CP, Ozua P, Newcomb PV, Savage P, Powell R, Rahamin J, Alderson D. Immunohistochemical detection of p53 and c-erbB-2 in oesophageal carcinoma; no correlation with prognosis. Eur J Surg Oncol 1997; 23(1):30-35.

[37] Lal P, Salazar PA, Ladanyi M, Chen B. Impact of Polysomy 17 on HER-2/neu immunohistochemistry in breast carcinomas without HER-/neu gene amplification. JMD 2003; 5; 3:155-159.

[38] Lauren P. The two histological main types of gastric carcinoma: diffuse and so-called intestinal type carcinoma. Acta Pathol Microbiol Scand 1965; 64:31-49.

[39] Eastmond DA, Schuler M, Rupa DS. Advantages and limitations of using fluorescence in situ hybridization for the detection of aneuploidy in interphase human cells. Mutat Res 1995;438:153-162.

[40] Lee- Análise de Concordância Kappa. 1995. Available at: http://www.lee.dante.br/pesquisa/kappa/index.html. Accessed January, 2005.

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

unesp

[42] Yasogawa Y, Takano Y, Okayasu I, Kakita A. The 5D4 antibody (anti-cyclin D1/D2) related antigen: Cytoplasmatic staining is correlated to the progression of gastric cancer. Pathol Int 1998; 48:717-722.

[43] Takano Y, Kato Y, van Diest PJ, Masuda M, Mitomi H, Okayasu I. Cyclin D2 overexpression and lack of p27 correlate positively and cyclin E inversely with a poor prognosis in gastric cancer cases. Am J Pathol 2000; 156:585-594.

[44] Schuring E. The involvement of the chromosome 11q13 region in human malignancies: cyclin D1 and EMS1 are two new candidate oncogenes – a review. Gene 1995; 159:83-96.

[45] Shinozaki H, Ozawa S, Ando N, Tsuruta H, Terada M, Ueda M, Kitajima M. Cyclin D1 amplification as a new predictive classification for squamous cell carcinoma of the esophagus, adding gene information. Clin Cancer Res 1996; 2:1155-1161.

[46] Leach FS, Elledge SJ, Sherr CJ, Willson JK, Markowitz S, Kinzler KW, Vogelstein B. Amplification of cyclin genes in colorectal carcinomas. Cancer Res 1993; 53(9):1986-1989.

[47] Gillett C, Fantl V, Smith R, Fisher C, Bartek J, Dickson C, Barnes D, Peters G. Amplification and overexpression of cyclin D1 in breast cancer detected by immunohistochemical staining. Cancer Res 1994; 54(7):1812-1817.

[48] Buckley MF, Sweeney KJ, Hamilton JA, Sini RL, Manning DL, Nicholson RI, deFazio A, Watts CK, Musgrove EA, Sutherland RL. Expression and amplification of cyclin genes in human breast cancer. Oncogene 1993; 8(8):2127-2133.

[49] Nishimoto IN, Pinheiro NA, Rogatto SR, Carvalho AL, Simpson AJ, Caballero OL, Kowalski LP. Cyclin D1 gene polymorphism as a risk factor for squamous cell carcinoma of the upper aerodigestive system in non-alcoholics. Oral Oncology 2004; 40: 604-610.

[50] Ishikawa T, Kobayashi M, Mai M, Suzuki T, Ooi A. Amplification of the c-erbB-2 (HER-2/neu) gene in gastric cancer cells. Detection by fluorescence in situ hybridization. Am J Pathol 1997;151(3):761-768.

[51] Hardisson D, Alvarez-Marcos C, Salas-Bustamante A, Alonso-Guervos M, Sastre N, Sampedro A. Numerical aberrations of chromosomes 8, 9, 11, and 17 in squamous cell carcinoma of the pharynx and larynx: a fluorescence in situ hybridization and DNA flow cytometric analysis of 50 cases. Oral Oncol 2004; 40(4):409-417.

[52] Cesar AC, Borim AA, Caetano A, Cury PM, Silva AE. Aneuploidies, deletion, and overexpression of TP53 gene in intestinal metaplasia of patients without gastric cancer. Cancer Genet Cytogenet. 2004; 153(2):127-132.

[53] Ross JS, McKenna BJ. The HER-2/neu oncogenes in tumors of the gastrointestinal tract. Cancer Invest 2001; 19:554-568.

[54] Allgayer H, Babic R, Gruetzner KU, Tarabichi A, Wilhelm S, Heiss MM. c-erbB-2 is of independent prognostic relevance in gastric cancer and is associated with the expression of tumor-associated protease systems. J Clin Oncol 2000; 18(11):2201-2209.

[55] Arteaga CL. Epidermal growth factor receptor dependence in human tumors: more than just expression? Oncologist 2002; 7;4: 31-39.

cm 1 2 3 4 5 6 7 unesp 11 12 13 14 15 16 17 18

[56] De-Vita VJR, Hellman S, Rosenberg SA. Cancer - Principles and Practice of Oncology, 5th ed. v.1: Philadelphia, New York: Lippincott-Raven, 1997; p.1154.

Table 1. Results of gene gain (amplification and polysomy) of the *CCND1* and *HER-2/neu* genes in normal and tumor tissues of esophagus and stomach.

Samples	Disomy (G/C= 0.9 – 1.0)	Gene Status			Total
		Amplification (G/C > 2.0)	Polysomy (>15%)	Total	
Normal Esophageal Mucosa					
CCND1	12 (100%)	0 (0%)	0 (0%)	0 (0%)	12 (100%)
HER-2/neu	11 (100%)	0 (0%)	0 (0%)	0 (0%)	11 (100%)
Esophageal Carcinoma					
CCND1	12 (30%)	18 (45%)	10 (25%)	28 (70%)	40 (100%)
HER-2/neu	24 (60%)	3 (7.5%)	13 (32.5%)	16 (40%)	40 (100%)
Normal Gastric Mucosa					
CCND1	14 (100%)	0 (0%)	0 (0%)	0 (0%)	14 (100%)
HER-2/neu	12 (100%)	0 (0%)	0 (0%)	0 (0%)	12 (100%)
Gastric Adenocarcinoma					
CCND1	29 (72.5%)	4 (10%)	6 (15%)	10 (25%)	40 (100%)
HER-2/neu	32 (80%)	2 (5%)	6 (15%)	8 (20%)	40 (100%)

G/C: gene-to-centromere ratio

Table 2. Results of overexpression of Cyclin D1 and Her-2/neu proteins in normal and tumoral tissues of esophagus and stomach.

Samples	Cyclin D1			Her-2/neu			
	(+)	(-)	Total	Negative	Low level	High level	Total
Normal Esophageal Mucosa	1 16.6%	5 83.3%	6 100%	6 100%	0 0%	0 0%	6 100%
Esophageal Carcinoma	15 37.5%	25 62.5%	40 100%	32 80%	3 7.5%	5 12.5%	40 100%
Normal gastric mucosa	0 0%	7 100%	7 100%	7 100%	0 0%	0 0%	7 100%
Gastric Adenocarcinoma	14 35%	26 65%	40 100%	34 85%	3 7.5%	3 7.5%	40 100%

Table 3. Association between gene gains by amplification and polysomy with Cyclin D1 and Her-2/neu proteins expression in esophageal carcinoma and gastric adenocarcinoma.

FISH	Esophageal Carcinoma				Gastric Adenocarcinoma			
	Cyclin D1 (+)	Cyclin D1 (-)	Her-2/neu (+)	Her-2/neu (-)	Cyclin D1 (+)	Cyclin D1 (-)	Her-2/neu (+)	Her-2/neu (-)
Amplification								
Yes	10 (25%)	8 (20%)	1 (2.5%)	2 (5.0%)	4 (10%)	0 (0%)	1 (2.5%)	1 (2.5%)
No	5 (12.5%)	17(42.5%)	4 (10%)	33 (82.5%)	10 (25%)	26 (65%)	2 (5.0%)	36 (90%)
Kappa	*0.333***		*0.172***		*0.340****		*0.360****	
Polysomy								
Yes	4 (10%)	6 (15%)	3 (7.5%)	10 (25%)	3 (7.5%)	3 (7.5%)	2 (5.0%)	4 (10%)
No	11 (27.5%)	19(47.5%)	2 (5.0%)	25 (62.5%)	11(27.5%)	23 (57.5%)	1 (2.5%)	33 (82.5%)
Kappa	*-0.176**		*0.186***		*0.114***		*0.383****	

* Value of *Kappa*<0: no agreement
** Value of *Kappa* =0-0.19: poor agreement
*** Value of *Kappa* =0.20-0.39: fair agreement
FISH: Fluorescence *in situ* hybridization

Table 4. Relationship between *CCND1* and *HER-2/neu* gene status and protein expression in esophageal carcinoma and gastric adenocarcinoma

Category	Esophageal carcinoma		Gastric adenocarcinoma	
	Gene status	**Overexpression**	**Gene status**	**Overexpression**
Cyclin D1 + / Her-2/neu +	12 (30%)	2 (5%)	3 (7.5%)	1 (2.5%)
Cyclin D1 + / Her-2/neu -	17 (42.5%)	14 (35%)	8 (20%)	13 (32.5%)
Cyclin D1 - / Her-2/neu +	4 (10%)	3 (7.5%)	5 (12.5%)	2 (5%)
Cyclin D1 -/ Her-2/neu -	7 (17.5%)	21 (52.5%)	24 (60%)	24 (60%)
Kappa	0.030**	0.010**	0.100**	-0.001*

* Value of *Kappa*<0: no agreement

** Value of *Kappa* =0-0.19: poor agreement

Tabela 5. Demographic and clinicopathological distribution of esophageal carcinoma patients in relation the *CCND1* and *HER-2/neu* gene status and protein levels.

Variables	Gene Status (FISH)				Overexpression (IHC)			
	CCND1		*HER-2/neu*		Cyclin D1		Her-2/neu	
	Amplification	Polisomy	Amplification	Polisomy	(+)	(-)	(+)	(-)
Gender								
Male	16 (40%)	8 (20%)	3 (7.5%)	12 (30%)	13(32.5%)	21 (52.5%)	4 (10%)	30 (75%)
Female	2 (5%)	2 (5%)	0 (0%)	1 (2.5%)	3 (7.5%)	3 (7.5%)	1 (2.5%)	5 (12.5%)
P value	*0.6726*	*0.7059*	*1.0000*	*0.6434*	*0.6678*		*1.0000*	
Age								
< 60	7 (17.5%)	4 (10%)	2 (5%)	5 (12.5%)	5 (12.5%)	8 (20%)	3 (7.5%)	10 (25%)
> 60	11 (27.5%)	6 (15%)	1 (2.5%)	8 (20%)	11(27.5%)	16 (40%)	2 (5%)	25 (62.5%)
P value	*0.5092*	*0.7004*	*0.2421*	*0.7215*	*1.0000*		*0.3065*	
Smoking								
Yes	16 (40%)	9 (22.5%)	3 (7.5%)	10 (25%)	15(37.5%)	21 (52.5%)	5 (12.5%)	31 (77.5%)
No	2 (5%)	1 (2.5%)	0 (0%)	3 (7.5%)	1 (2.5%)	3 (7.5%)	0 (0%)	4 (10%)
P value	*0.6726*	*1.0000*	*1.0000*	*0.6622*	*0.6376*		*1.0000*	
Alcohol								
Yes	15 (37.5%)	6 (15%)	2 (5%)	10 (25%)	9 (22.5%)	22 (55%)	4 (10%)	27 (67.5%)
No	3 (7.5%)	4 (10%)	1 (2.5%)	3 (7.5%)	7 (17.5%)	2 (5%)	1 (2.5%)	8 (20%)
P value	*0.2863*	*0.4177*	*1.0000*	*0.7159*	*0.0175**		*1.0000*	
Histological Grade								
Well differentiated	16 (40%)	7 (17.5%)	1 (2.5%)	12 (30%)	12 (30%)	20 (50%)	5 (12.5%))	27 (67.5%)
Poorly Differentiated	2 (5%)	3 (7.5%)	2 (5%)	1 (2.5%)	4 (10%)	4 (10%)	0 (0%)	8 (20%)
P value	*0.6726*	*0.1528*	*0.0536*	*0.6434*	*0.6975*		*0.5628*	
Survival								
Dead	8 (20%)	6 (15%)	3 (7.5%)	7 (17.5%)	9 (22.5%)	13 (32.5%)	4 (10%)	18 (45%)
Alive	10 (25%)	4 (10%)	0 (0%)	6 (15%)	7 (17.5%)	11 (7.58%)	1 (2.5%)	17 (42.5%)
P value	*0.5254*	*0.7209*	*0.2327*	*1.0000*	*1.0000*		*0.3555*	

*Statistical significance was demonstrated with $P<0.05$ (Fisher's exact test);
FISH: Fluorescence in situ hybridization
IHC: Immnohistochemical



Tabela 6. Demographic and clinicopathological distribution of gastric adenocarcinoma patients in relation the *CCND1* and *HER-2/neu* gene status and protein levels.

Variables	Gene Status (FISH)				Overexpression (IHC)			
	CCND1		*HER-2/neu*		Cyclin D1		Her-2/neu	
	Amplification	Polisomy	Amplification	Polisomy	(+)	(-)	(+)	(-)
Gender								
Male	4 (10%)	3 (7.5%)	2 (5%)	3 (7.5%)	12 (40%)	18 (45%)	2 (5%)	28 (70%)
Female	0 (0%)	3 (7.5%)	0 (0%)	3 (7.5%)	2 (5%)	8 (20%)	1 (2.5%)	9 (22.5%)
P value	*0.5588*	*0.1528*	*1.0000*	*0.1528*	*0.4457*		*1.0000*	
Age								
< 60	1 (3%)	4 (10%)	1 (2.5%)	4 (10%)	8 (20%)	18 (45%)	2 (5%)	24 (60%)
> 60	3 (7.5%)	2 (5%)	1 (2.5%)	2 (5%)	6 (15%)	8 (20%)	1 (2.5%)	13 (32.5%)
P value	*1.0000*	*0.1591*	*1.0000*	*0.1591*	*0.5013*		*1.0000*	
Smoking								
Yes	4 (10%)	1 (2.5%)	1 (2.5%)	1 (2.5%)	12 (30%)	14 (35%)	1 (2.5%)	25 (62.5%)
No	0 (0%)	5 (12.5%)	1 (2.5%)	5 (12.5%)	2 (5%)	12 (40%)	2 (5%)	12 (40%)
P value	*0.1079*	*0.0848*	*1.0000*	*0.0734*	*0.0807*		*0.2763*	
Alcohol								
Yes	4 (10%)	0 (0%)	1 (2.5%)	1 (2.5%)	10 (25%)	11(27.5%)	1 (2.5%)	20 (50%)
No	0 (0%)	6 (15%)	1 (2.5%)	5 (12.5%)	4 (10%)	15 (37.5%)	2 (5%)	17 (42.5%)
P value	*0.0260**	*0.0295**	*1.0000*	*0.2156*	*0.1046*		*0.5962*	
Histological Grade								
Diffuse	3 (7.5%)	5 (12.5%)	2 (5%)	3 (7.5%)	8 (20%)	18 (45%)	2 (5%)	12 (40%)
Intestinal	1 (2.5%)	1 (2.5%)	0 (0%)	3 (7.5%)	6 (15%)	8 (20%)	1 (2.5%)	25 (62.5%)
P value	*1.0000*	*0.3990*	*0.5333*	*0.6456*	*0.5013*		*0.2763*	
***H. pylori* infection**								
Positive	1 (2.5%)	1 (2.5%)	1 (2.5%)	1 (2.5%)	6 (15%)	17 (42.5%)	2 (5%)	15(37.5%)
Negative	3 (7.5%)	5 (12.5%)	1 (2.5%)	5 (12.5%)	8 (20%)	9 (22.5%)	1 (2.5%)	22 (55%)
P value	*1.0000*	*0.3990*	*1.0000*	*0.3990*	*0.1978*		*0.5647*	
Survival								
Dead	1 (2.5%)	2 (5%)	1 (2.5%)	1 (2.5%)	2 (5%)	11(27.5%)	2 (5%)	11(27.5%)
Alive	3 (7.5%)	4 (10%)	1 (2.5%)	5 (12.5%)	12 (40%)	15(37.5%)	1 (2.5%)	26 (65%)
P value	*0.7778*	*0.3990*	*0.5154*	*0.6479*	*0.0897*		*0.2421*	

*Statistical significance was demonstrated with $P<0.05$ (Fisher's exact test);
FISH: Fluorescence in situ hybridization
IHC: Immnohistochemical



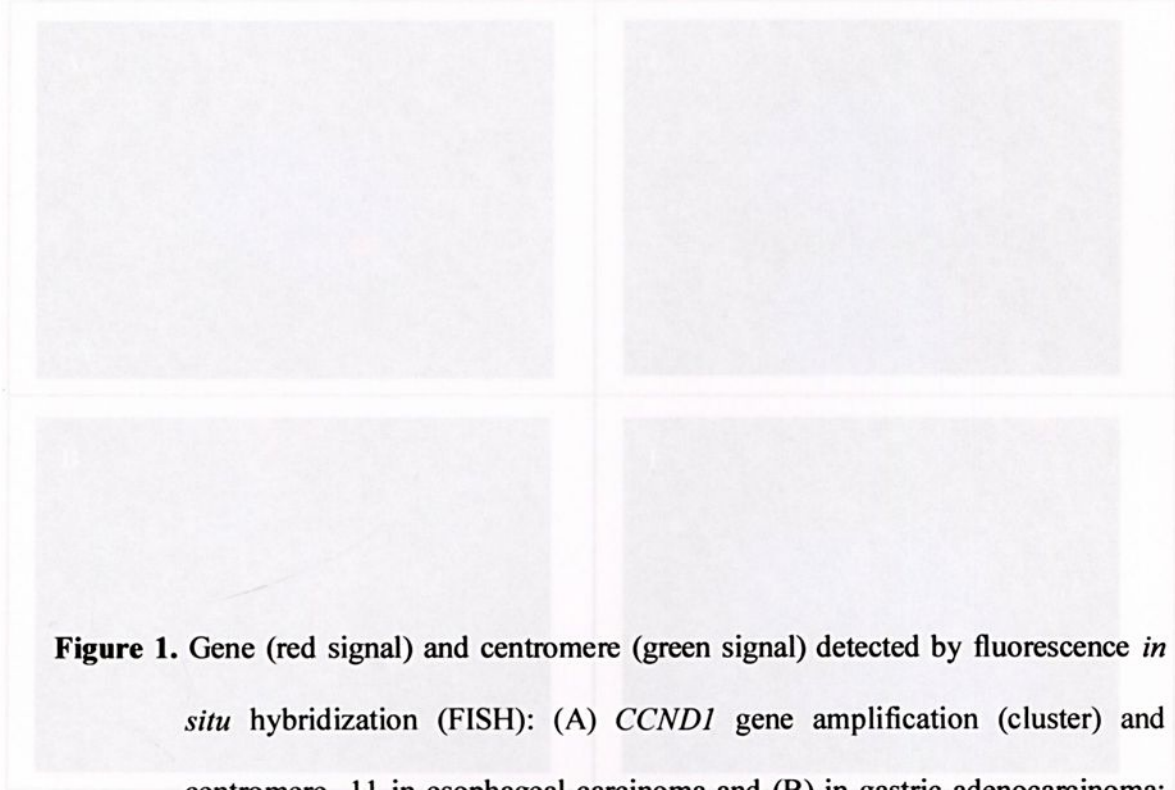
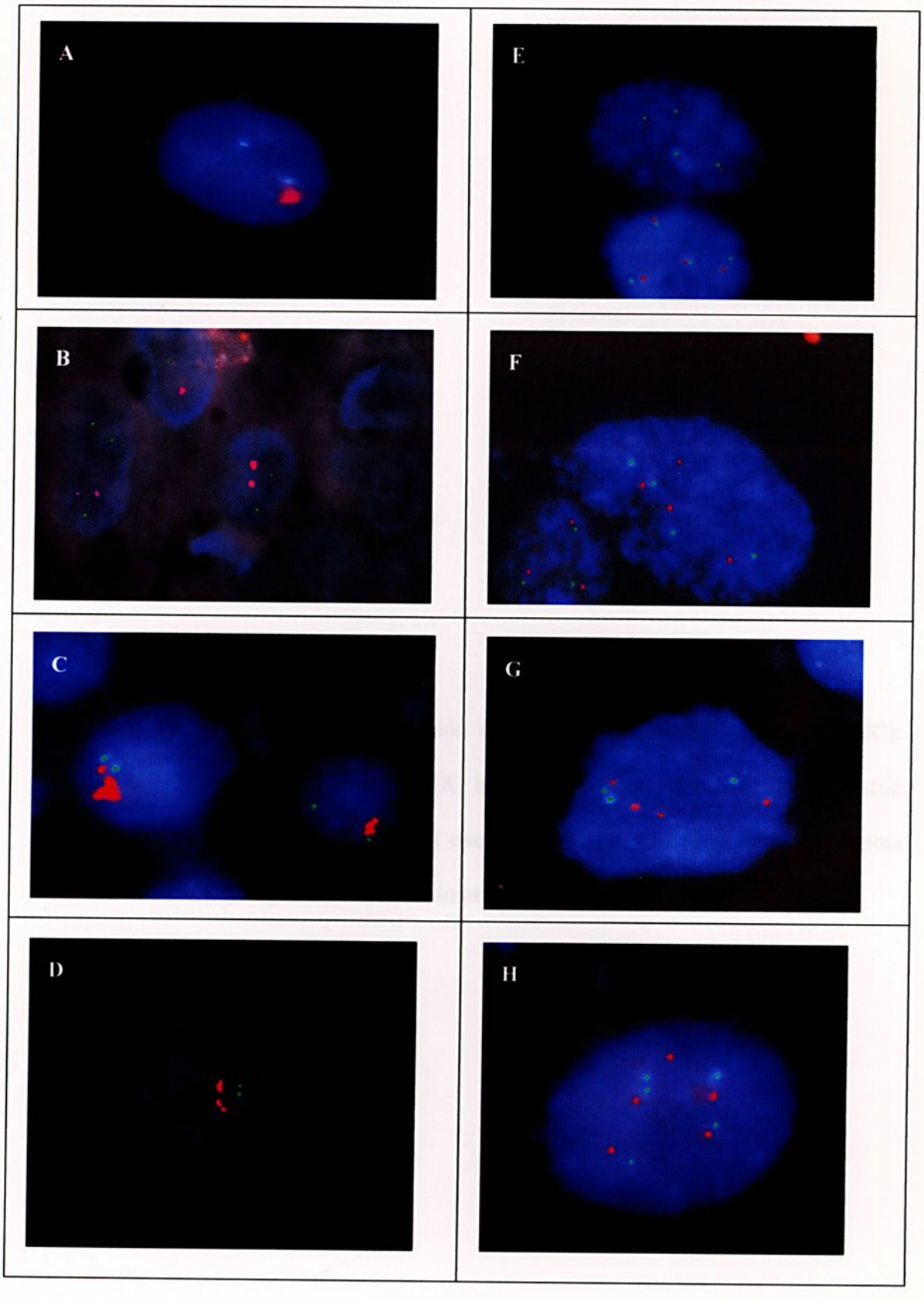


Figure 1. Gene (red signal) and centromere (green signal) detected by fluorescence *in situ* hybridization (FISH): (A) *CCND1* gene amplification (cluster) and centromere 11 in esophageal carcinoma and (B) in gastric adenocarcinoma; (C) *HER-2/neu* gene amplification (cluster) and centromere 17 in esophageal carcinoma and (D) in gastric cancer; (E) polysomy of chromosome 11 and *CCND1* gene gain in esophageal carcinoma and (F) in gastric cancer; (G) polysomy of chromosome 17 and *HER-2/neu* gene gain in esophageal carcinoma and (H) in gastric adenocarcinoma.

cm 1 2 3 4 5 6 7 8 unesp 12 13 14 15 16 17 18 19


A
E
B
F
C
G
D
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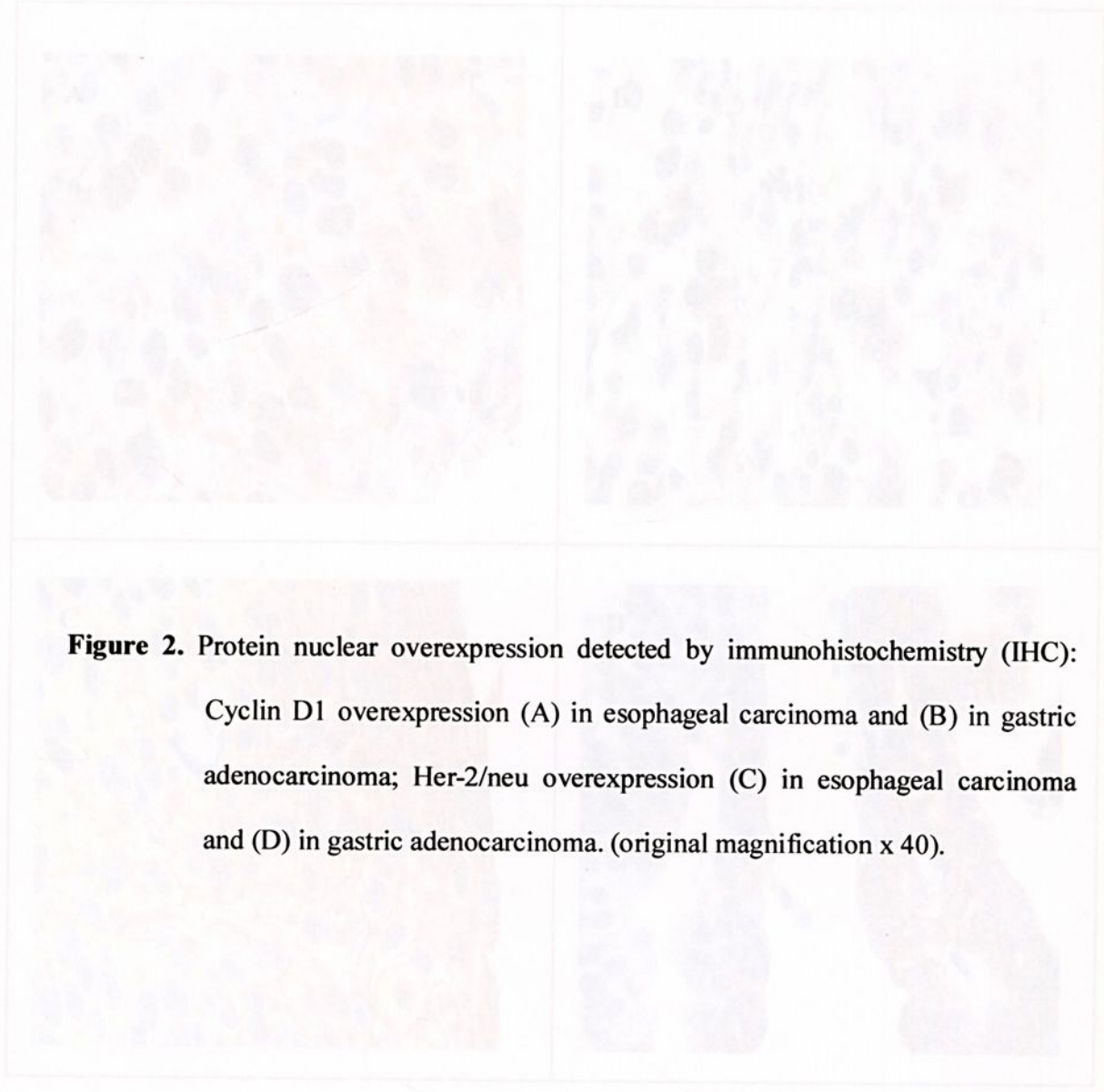
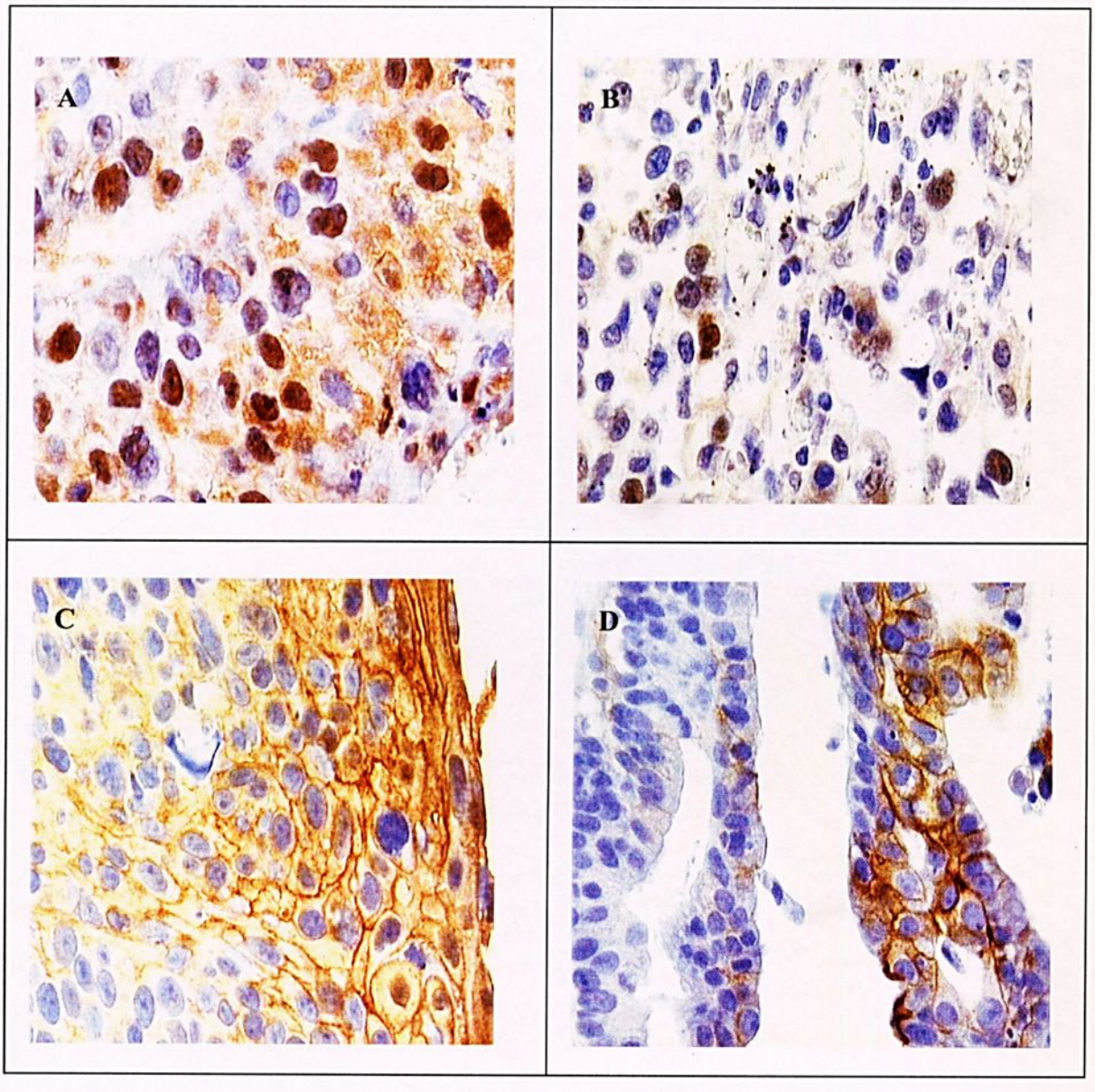


Figure 2. Protein nuclear overexpression detected by immunohistochemistry (IHC): Cyclin D1 overexpression (A) in esophageal carcinoma and (B) in gastric adenocarcinoma; Her-2/neu overexpression (C) in esophageal carcinoma and (D) in gastric adenocarcinoma. (original magnification x 40).

cm 1 2 3 4 5 6 7 8 unesp 12 13 14 15 16 17 18 19


A
B
C
D

Artigo Original:

CCND1 and HER-2/neu gene position within chromosome territories and association with amplification and overexpression in esophageal and gastric carcinomas.

CCND1 AND *HER-2/neu* GENE POSITION WITHIN CHROMOSOME TERRITORIES AND ASSOCIATION WITH AMPLIFICATION AND OVEREXPRESSION IN ESOPHAGEAL AND GASTRIC CARCINOMAS

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Running title: *CCND1* and *HER-2/neu* territorial positioning in esophageal and gastric cancers.

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Abstract

Individual interphase chromosomes have been shown to occupy discrete regions of the nuclear volume called chromosome territories (CT), and their active genes are preferentially positioned on the surfaces of CTs, where they are accessible to the transcriptional machinery. We analyzed by FISH the *CCND1* and *HER-2/neu* gene positions within the CTs and their relationship with gene amplification and protein overexpression in esophageal and gastric cancers. Both genes were preferentially positioned at the periphery (mean frequency of 50-83%) of the CTs, both in normal mucosa and in tumor tissues of the esophagus and stomach. However, this positioning revealed no exact association with the amplification or protein overexpression status of these genes, although, in esophageal carcinoma *Kappa* statistics showed a moderated agreement between amplification of the *CCND1* gene (*Kappa*=0.400) and its location within the CT, as well as with overexpression of the correspondent protein (*Kappa*=0.444). Thus, our results suggest that the gene positioning in the interphase chromosome does not depend only on the transcriptional activity of the gene, but also on other factors acting together, such as dense-gene, chromosome size, chromatin structure, level and stability of its expression and tissue specificity of its expression.

Key words: *CCND1*, *HER-2/neu*, chromosome territories, cancer.

Introduction

Various studies focusing on the molecular components of DNA replication, transcription, RNA processing, DNA repair, and on the nuclear matrix (Berezney et al., 1995; Lamond; Earnshaw, 1998) have demonstrated that the interphase nucleus is structurally and functionally compartmentalized into regions that contain factors involved in RNA synthesis, processing and transport (Park; De Boni, 1998).

The chromosomes have shown to maintain their identity during the entire cell cycle and did not indicate movement during interphase (Zink et al., 1998; Edelmann et al., 2001), thus occupying discrete, non-overlapping regions of the nuclear volume called chromosome territories (CT) or chromosome domains (CD) (Cremer et al., 1993; Visser; Aten, 1999). Within these territories, active genes are positioned at the surface, where they are accessible to the transcriptional machinery and splicing factors, and where RNA transcripts could be released directly into the CT for transportation to the nuclear pores (Park; De Boni, 1998; Volpi et al., 2000).

Fluorescence *in situ* hybridization (FISH) has proved to be an effective tool for investigators in researching interphase chromosome organization and in understanding the spatial compartmentalization of inactive and active genes. Particular emphasis has been placed on the question of whether the transcriptional activity of genes can affect their territorial positioning and, indeed, whether active genes can leave the territory altogether (Bartová et al., 2000; Williams, 2003). Firstly, Kurz et al. (1996) demonstrated that in muscle cells, fibroblasts and HeLa cells, the *DMD*, *HBB* and *MYH7* genes (but not non-coding regions) are preferentially located in the periphery of their respective CTs, regardless of their activity. Extending this analysis, other studies have reported the

preferential positioning of different genes within CTs in some cell types, with or without an association with transcriptional activity (Park; De Boni, 1998; Dietzel et al., 1999; Bartová et al., 2000; Volpi et al., 2002).

In contrast, Mahy et al. (2002) found that the *WAGR* locus at 11p13 (associated with Wilm's tumor aniridia, genitourinary anomalies, mental retardation syndrome and containing the genes *WT1, RCN, PAX6* and *PAXNEB*) is located well within the painted chromosome 11 territory and, more importantly, that transcriptional activation of these genes did not result in their relocation to the territory surface. Thus, they suggested that active genes might be found not only at the surface, but also within the territory, on the surface of the condensed chromatin subdomains that line the invaginating interchromatin channels (Williams, 2003). Such analyses reveal that the positions of gene loci with respect to the territory are not absolute, but can represent a statistically significant percentage.

It is important to note that only a limited number of genes/loci have been examined in terms of chromosome territory and expression. It is therefore unclear if all genes behave similarly (Kosak; Groudine, 2002) and, moreover, there is evidence that genes can be found anywhere within a chromosome territory, regardless of their transcriptional activity (Parada et al., 2002). The core question is whether particular types or classes of genes demonstrate a conserved territorial position.

Given that the positioning of transcriptionally active sequences in the periphery of chromosome territories has been controversial, the aims of this study were: to evaluate by dual-color FISH the spatial intranuclear distribution of the *CCND1* and *HER-2/neu* genes within their respective chromosome 11 and 17 territories, in esophageal carcinoma and gastric adenocarcinoma, and to correlate the results with the amplification

and overexpression status of both the *CCND1* and the *HER-2/neu* genes. Esophageal and gastric cancers were chosen as models due to indications of increased gene amplification and overexpression of the *CCND1* and *HER-2/neu* sequences.

Material and methods

Patients

A total of 20 specimens (fresh tumor tissue) were obtained before any chemotherapy and/or radiotherapy from 10 patients with esophageal squamous cell carcinoma (10 males) and 10 patients with gastric adenocarcinoma (8 males and 2 females). All patients were diagnosed and operated on by the Department of Surgery of *Hospital de Base*, in São José do Rio Preto, SP, Brazil. The mean age of the patients with esophageal carcinoma was 58.2 years (range 46-65 years), and that of the patients with gastric adenocarcinoma was 56 years (range 38-82 years). The esophageal tumors were graded according to the World Health Organization: 8 tumors were classified as moderately differentiated, 1 as adenocarcinoma, and 1 as carcinoma *in situ*. The gastric adenocarcinomas were classified by characteristic histological features (Laurén, 1965) into diffuse (7 specimens) or intestinal (3 specimens) types. Biopsies (5 normal esophageal mucosae and 5 normal gastric mucosae) obtained from the same patients, but removed from distant surgery areas, were used as controls.

Written informed consent was obtained from all patients, and the project was approved by the National Research Ethics Committee.

Cell culture

Esophageal and gastric cancer and normal mucosa tissues were cultured in α-MEM medium, supplemented with 10% fetal calf serum, 1mM sodium pyruvate, 0.1mM non-essential aminoacids and penicillin/streptomycin, in an incubator at 37°C with a humidified atmosphere containing 5% CO_2. After about 15-20 days, the cells were synchronized in the G_0/G_1 phase by serum deprivation for 48 hours. The cells were then detached by trypsin, washed by centrifugation in α-MEM medium, and incubated in a hypotonic solution (0.075M KCl) for 20 minutes at 37°C. Then the cells were fixed by standard cytogenetic procedure in three changes of fresh 3:1 methanol:acetic acid and dropped onto clean glass slides. Although methanol:acetic acid generates a looser chromatin packing, allowing only bi-dimensional analysis, it has been used successfully to study large-scale chromatin organization *in situ*, with results comparable to paraformaldehyde fixation and three-dimensional preservation of the nuclear structure (Yokota et al., 1997; Bartová et al., 2000; Volpi et al., 2000).

Fluorescence in situ hybridization (FISH)

Chromosome territories

Dual-color FISH assays were performed on cell suspensions obtained from the cell culture, using the LSI Cyclin D1 sequence labeled in Spectrum Orange and Whole Chromosome Painting (WCP) 11 labeled in Spectrum Green (Vysis), and the HER-2/neu kit (Q-bioGene), including the *HER-2/neu* sequence probe labeled with rhodamine and WCP 17 labeled with fluorescein, following the instructions given by the manufacturer.

Briefly, the slides were treated with 70% acetic acid at room temperature for 5 min. Then, a solution containing the Cyclin D1 and WCP11 or HER-2/neu and WCP17 probes was applied to the specimens and co-denatured at 80°C for 5-8 min. Afterwards, the slides were hybridized overnight (16 to 18 hours) at 37°C in a moist chamber. When post-hybridization had washed out, the slides were counterstained with DAPI (4', 6-diamino-2-phenylindole). After post-hybridization washes, both chromosomal territories (#11 and #17) were visualized in green and the genes (*CCND1* and *HER-2/neu*) in red.

The slides were evaluated in an Olympus BX60 microscope. The positions of FISH signals from individual regions (*CCND1* and *HER-2/neu* genes) of chromosomes 11 and 17 were analyzed in relation to the respective chromosome territories, defined by chromosomes 11 and 17-specific paint, in about 200 territories, following a previously reported protocol (Volpi et al., 2000). In polysomic nuclei, each territory was evaluated separately. In this analysis, the gene signal was named "internal" if it was inside the painted domain and did not touch the border of the painted domain; "peripheral" if it touched the border of the painted domain, and "external" if it was outside the painted domain without touching its border. In our experiments, we have grouped together under the definition "peripheral" both the "peripheral" and the "external" hybridization patterns.

Gene amplification

FISH assays were performed on cell suspension obtained of non-cultured fresh tumor and normal tissues using Cyclin D1 probe kit (Vysis), including the LSI Cyclin D1 sequence labeled in Spectrum Orange with the chromosome 11 centromere sequence labeled in Spectrum Green and the HER-2/neu kit (Q-bioGene), including the probe HER-

2/neu sequence labeled with Rhodamine with the chromosome 17 centromere labeled in Fluorescein, following the instructions given by the manufacturer. All specimens were mechanically and enzymatically disagregated with pepsin 0.008% and single cell suspension obtained was treated with hypotonic solution (0.075M KCl) and fixed in methanol-acetic acid (3:1) overnight. After washes in 60% acetic acid, the resulting cell suspension was dropped onto microscope slides. Briefly, the slides were treated with a acetic acid 70% at room temperature for 5 min. Then, a solution containing the Cyclin D1 or HER-2/neu probes were applied to the specimens and co-denatured at 80°C for 5-8 min, according to described above for chromosome territories.

The slides were also evaluated in a Olympus BX60 microscope. The number of signals from the chromosome 11 centromere and of Cyclin D1 gene were scored together, and the signal numbers of chromosome 17 centromere was scored with those of *HER-2/neu* gene in about 250 nuclei in accordance to the criteria described by Eastmond et al. (1995). The gene-to-centromere (G/C) ratio was calculated for each case; that is, the total number of gene signals observed was divided by the total number of centromere signals. For each FISH probe tested, the status of the chromosome used as control was classified as disomy, or polysomy when, respectively, on average, 2 or 3, or more copies scored per nucleus. Gene gains was considered either by amplification or high-balanced polisomy that is recognized as overrepresentation (Patrapim et al., 2004). Gene amplification assessed by multiple copies of the gene in any chromosomal status was classified as high level when gene/centromere ratio was > 2.0 or with clustered copies (groups of many copies of the gene), while high-balanced polisomy was considered as positive only in the cases with high frequencies (>15%).

Immunohistochemistry (IHC)

Tumor material consisted of paraffin-embedded specimens fixed in 10% formalin. Sections were cut at 5µm, dried for 1 h at 65°C. After deparaffinization and rehydration of the sections, antigen retrieval was performed in a solution of Tris-EDTA pH 9.0 for Cyclin D1 and citrate 10mM, pH 6.0 for Her-2/neu antibody. All further steps were performed using an automated system (Cadenza). Endogenous peroxidases were inhibited by 20 min incubation in 3% H_2O_2. Then, sections were incubated with the monoclonal Cyclin D1 or Her-2/neu antibodies (Dako), used at 1:100 and 1:200 dilution respectively, overnight at room temperature. Immunodection was achieved using the kit NOVOSTATIN Super ABC (NOVOCASTRA), for 35 min at room temperature. Then, sections were incubated with diaminobenzidine for 5 min and counterstained for 15 sec with hematoxylin. Amigdala and breast carcinomas were used as positive control for Cyclin D1 and Her-2/neu antibodies, respectively.

All specimens were revised by a single pathologist and only IHC status was evaluated. For cyclin D1 immunostaining were counted about 500 nuclei. The positivity index was defined as greater than 30% and 20% of nuclei showing strong cyclin D1 immunostaining in esophageal carcinomas and gastric adenocarcinomas, respectively, according to mean frequencies observed in the respectives normal mucosas. The nuclear membrane staining intensity and pattern were considered for scoring according to the Food and Drug Administration approved criteria for HER-2/neu antibody: 0, no staining or membrane staining observed in less than 10% of tumor cells; 1+, partial membrane staining in more than 10% of tumor cells and membrane staining not circumferential; 2+, circumferential, weak to moderate staining observed in more than 10% of tumor cells; and

3+, strong circumferential membrane staining observed in more than 10% of tumor cells. Scores of 2+ and 3+ were considered positive. To both proteins were not considered cytoplasmatic immunostaining. Areas that were poorly preserved, crushed, cauterized, folded, or retracted were specifically avoided.

Statistics

The ANOVA statistical test using Statidisk computer software program, was conducted to compare mean frequencies of gene position between normal and tumoral tissues with arcsen $(p)^{1/2}$ transformation. The level of significance was set at $P<0.05$.

Kappa statistics was conducted to examine the agreement between gene location in the CTs and gene amplification or overexpression levels for the two molecular targets. *Kappa* statistic were performed using website www.lee.dante.br/pesquisa/kappa/index.html.

Results

The results of each specimen of normal and tumor tissues of the esophagus and stomach, respectively, analyzed for location of genes within their respective chromosome territories, amplification and overexpression of the *CCND1 and HER-2/neu* genes, are summarized in Tables 1 and 2. In all experiments, the peripheral and external patterns were added together in the peripheral category, in view of the low frequencies of the external position observed in the majority of cases. Regarding the *CCND1* gene, about 50% of the signals were found to be present either inside (Figure 1A) or at the periphery of

the painted chromosome 11 territory, in normal mucosa of both the esophagus and the stomach, while in esophageal and gastric carcinomas a higher frequency was observed in the periphery of the CT (60.55 and 83.73%, respectively) (Figure 1B). In fact, the ANOVA test showed a significant difference between the mean frequencies of the *CCND1* gene positions only between the groups of normal mucosa and gastric cancer ($P<0.0001$). Interestingly, for the *HER-2/neu* gene a different pattern was found (Figure 1C), since about 60% of the signals were located in a peripheral position of the 17 chromosome territory, in the esophageal carcinoma, as well as in the normal gastric mucosa and in the gastric adenocarcinoma samples, whereas in the normal esophageal mucosa it was found preferentially in an internal position (66.54%). So, statistically significant differences were found between both the groups of esophageal ($P=0.0203$) and gastric cancer ($P=0.0017$) and the respective groups of normal mucosa. In none of these cases a large external chromatin loop was observed, but in some of those with amplification a larger signal was evidenced in a cluster outside the chromosome territory (Figure 1D).

The association of the *CCND1* and *HER-2/neu* gene location within the respective chromosome territories with gene amplification and overexpression of these two molecular targets is shown in Tables 3 and 4 for esophageal carcinoma and gastric adenocarcinoma. According to *Kappa* statistics, an exact association between these parameters was not found in either esophageal or gastric cancers, but only a moderated agreement between amplification of the *CCND1* gene and an internal or peripheral position of this gene within its respective CT in esophageal carcinoma (*Kappa*=0.400). Regarding overexpression, a moderate and poor agreement of Cyclin D1 and Her-2/neu protein

expression with gene position was also observed in esophageal carcinoma (*Kappa*=0.444 and *Kappa*=0.194, respectively).

Although we did not investigate the spatial distribution of the 11 and 17 chromatin CTs in the nuclei, we observed that both these chromosome territories were located in a median position in about 60-80% of the nuclei of both normal and tumor tissues, except for gastric adenocarcinoma, in which these CTs were most commonly positioned in the periphery of the nuclei (range 61-72%).

Discussion

Individual chromosomes have been shown to occupy discrete regions of the nuclear volume, and their active genes are positioned at the surface of chromosome territories, where they are accessible to the transcriptional machinery (Cremer et al., 1993; Visser; Aten, 1999; Volpi et al., 2000). This model appears to be true at least for some chromosomes, but studies about gene positioning in different cell types are still scarce and the results are conflicting. Understandably, it is therefore unclear if all genes behave in the same manner.

The aim of this study was to analyze interphase chromosomes, in an attempt to understand the relationship between the position of a gene within its chromosome territory and its activity. As a model system, we examined the *CCND1* and *HER-2/neu* genes in normal mucosa and tumor tissue of the esophagus and stomach using a FISH assay, because these genes have been shown to be amplified and/or overexpressed in these two tumor types (Takehana et al., 2002; Shiomi et al., 2003; Dahlberg et al., 2004; Manoel-Caetano et al., 2004).

cm 1 2 3 4 5 6 7 8 unesp 12 13 14 15 16 17 18 19

In this study, the *CCND1* and *HER-2/neu* genes were mainly detected near the periphery (peripheral and external positions) of their chromosome territories, in both esophageal and gastric cancers, and also in their respective normal mucosae.

Other studies have observed a peripheral position of some genes within their CTs associated with transcriptional activity. For example, the *ANT-2* gene, subject to X inactivation, has been shown to reside at the periphery of the active X territory, but inside the inactive X territory, where it is silenced (Dietzel et al., 1999). In muscle cells, fibroblasts and HeLa cells, Kurz et al. (1996) observed that about of 73-88% of the *DMD, MYH7* and *HBB* genes were in peripheral positions within their chromosome territories, but the localization was independent from the activity of the respective genes, while a non-coding DNA segment of chromosome 11 was located randomly or predominantly inside the CT. These authors also showed that genes do not have to be confined to the interior of the territory to be in an inactive state. Thus, the organization of genes in the CTs must be characteristic for the genes, but independent from their activity status.

More recently, Volpi et al. (2000) demonstrated that the major human histocompatibility complex (MHC) region displays a cell- and activity-dependent organization in a large loop emanating from the territory of chromosome 6. This finding was related to the number of active genes in the MHC region. In human breast cancer cells, thin chromatin protrusions carrying amplified *HER-2/neu* sequences, which extend from the painted chromosome 17 territory, have also been found (Park; De Boni, 1998). On the other hand, Mahy et al. (2002) observed that the *WAGR* locus (11p13) was located well within the painted chromosome 11 territory, and that transcriptional activation of this gene

did not result in its relocation to the territory surface. Later on, Roix et al. (2003) observed that in human lymphomas the *CCND1* gene frequently localizes near the nuclear interior, whereas others (c-*MYC*, *BCL6* and *IGK*) are preferentially located near the periphery. These authors suggested that human gene loci frequently have non-random and gene-specific distribution patterns in the interphase nucleus. These observations seem to indicate that active genes might be found not only at the surface of the territory, but also within the territory. Chromosome territories are permeable to proteins and contain a large accessible internal surface created by a meshwork of interconnected channels (Parada; Misteli, 2002), thus there are no significant zones of exclusion within the nucleus.

Although a preferential positioning of *CCND1* and *HER-2/neu* sequences at the periphery of the CTs has been observed in two tumor types, our data did not reveal an exact relationship between the position and the amplification and overexpression status of these genes. To our best knowledge, this is the first study that evaluated the association between the location of the *CCND1* and *HER-2/neu* oncogenes and their amplification and overexpression in esophageal and gastric cancers. In their previous report on breast tumor cell lines, Park; De Boni (1998) did not relate the positioning of the *HER-2/neu* gene with alterations in the copy number of chromosome 17 either. As in our sample, tumors with amplification of the *HER-2/neu* gene presented non-uniformity in signal size, suggesting possible cluster. However, we did not commonly observe elongated protrusions of the signal extending outside the chromosome 17 territory, as observed by those authors. In addition, Kurz et al. (1996) also observed that the positioning of *DMD*, *MYH7* and *HBB* sequences was independent from the activity of the genes assessed by mRNA amplification.



The *CCND1* and *HER-2/neu* genes, mapped to chromosome bands 11q13 (Motokura et al., 1993) and 17q11.2-q12 (Yamamoto et al., 1986), respectively, are both located in an R-band (early-replication region), believed to contain more transcriptionally active genes than G-bands (Kurz et al., 1996; Volpi et al., 2000). Thus, our findings reinforce the hypothesis that R-bands are predominantly located outside the CTs (Volpi et al., 2000), so sequences in R-bands would be expected to locate preferentially in the periphery of the chromosome territories, as observed in the current study.

We had no measurement standard of the radial arrangement of chromosome 11 and 17 territories in relation to the nucleus, but we observed that both chromosomes 11 (144Mb) and 17 (92Mb), which present a similar gene density (12 genes/Mb and 15 genes/Mp, respectively, in www.ncbi.nlm.nih.gov) were preferentially positioned in the median region of nuclei of both normal and tumor tissues. Only in gastric adenocarcinoma these chromosomes were preferentially positioned towards the periphery of the nucleus.

The radial position of chromosomes in the nucleus is still a matter under discussion. In breast tumor cells, chromosome 17 was not found to occupy a preferential position within the nuclear space (Park; De Boni, 1998). However, several sets of experiments indicate that the radial position of a given chromosome territory is correlated with its size, its gene density and its replication timing. Generally, the gene-rich and early-replicating CTs are typically positioned in the nuclear interior, while the gene-poor and later-replicating CTs are preferentially located at the periphery of the nucleus (Chevret et al., 2000; Tanabe et al., 2002). Moreover, the radial positioning according to size allows to predict that large chromosomes will be positioned towards the periphery and small chromosomes towards the interior (Sun, 2000). For example, in human lymphocytes, the



gene-poor chromosome 18 is observed to locate preferentially toward the nuclear periphery, whereas the gene-rich chromosome 19 is rather seen in the nuclear interior (Croft et al., 1999). Cremer et al. (2003) also obtained similar results in different normal and tumoral cell types, when they analyzed the radial distribution of the chromosome 18 and 19 chromatin. However, compared with nuclei of normal cells, most tumor cell lines showed a higher fraction of nuclei with inverted positions of these chromosomes, that is, CT 18 located more internally than CT 19.

Regardless of results which strongly support the hypothesis that genes are preferentially positioned in the periphery of the CTs for transcription to occur, other features such as gene density, DNA transcriptional level in G-band and R-band (gene-rich) regions, chromatin structure, gene characteristics, chromosomal structure, expression level and stability and tissue expression specificity, should also be considered (Volpi et al., 2000; Kosak; Groudine, 2002).

In conclusion, despite the inherent restrictions of the analysis procedure, our results suggest a preferential positioning of the *CCND1* and *HER-2/neu* genes in the periphery of chromosome territories, both in normal mucosa and in tumoral tissues of the esophagus and stomach, but without an exact association with amplification or overexpression of these genes. These findings emphasize the idea that other factors beyond gene transcriptional activity must be acting together. Thus, further studies are needed to elucidate the relevance of gene positioning in interphase chromosome territories and differential gene expression.

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References

Bartová E, Kozubek S, Kozubek M. et al. (2000) Nuclear topography of the c-myc gene in human leukemic cells. Gene. 22;244(1-2): 1-11.

Berezney R, Mortillaro MJ, Ma H, Wei X, Samarabandu J. (1995) The nuclear matrix: a structural milieu for genomic function. Int Rev Cytol. 162A: 1-65. Review.

Chevret E, Volpi EV, Sheer D. (2000) Mini review: form and function in the human interphase chromosome. Cytogenet Cell Genet. 90(1-2):13-21. Review.

Cremer T, Kurz A, Zirbel R. et al. (1993) Role of chromosome territories in the functional compartmentalization of the cell nucleus. Cold Spring Harb Symp Quant Biol. 58: 777-792.

Cremer M, Kupper K, Wagler B et al. (2003) Inheritance of gene density-related higher order chromatin arrangements in normal and tumor cell nuclei. J Cell Biol. 1;162(5): 809-820.

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Croft JA, Bridger JM, Boyle S, Perry P, Teague P, Bickmore WA. (1999) Differences in the localization and morphology of chromosomes in the human nucleus. J Cell Biol. 14;145(6): 1119-1131.

Dahlberg PS, Jacobson BA, Dahal G. et al (2004) ERBB2 amplifications in esophageal adenocarcinoma. Ann Thorac Surg 78(5): 1790-800.

Dietzel S, Schiebel K, Little G. (1999) The 3D positioning of ANT2 and ANT3 genes within female X chromosome territories correlates with gene activity. Exp Cell Res. 1;252(2): 363-375.

Eastmond DA, Schuler M, Rupa DS. (1995) Advantages and limitations of using fluorescence in situ hybridization for the detection of aneuploidy in interphase human cells. Mutat Res 438: 153-162.

Edelmann P, Bornfleth H, Zink D, Cremer T, Cremer C. (2001) Morphology and dynamics of chromosome territories in living cells. Biochim Biophys Acta. 1551(1): M29-M39.

Kosak ST, Groudine M. The undiscovered country: chromosome territories and the organization of transcription. (2002) Dev Cell. 2(6): 690-692.

Kurz A, Lampel S, Nickolenko JE et al. (1996) Active and inactive genes localize preferentially in the periphery of chromosome territories. J Cell Biol. 135(5):1195-1205.

Lamond AI, Earnshaw WC. (1998) Structure and function in the nucleus. Science 280: 547-553.

Lauren P. (1965) The two histological main types of gastric carcinoma: diffuse and so-called intestinal type carcinoma. Acta Pathol Microbiol Scand 64: 31-49.

Mahy NL, Perry PE, Gilchrist S, Baldock RA, Bickmore WA. (2002) Spatial organization of active and inactive genes and noncoding DNA within chromosome territories. J Cell Biol. 13;157(4): 579-89. Epub 2002 May 06.

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

Motokura T, Arnold A. (1993) Cyclin D1 and oncogenesis. Current Opin Genet Devel. 3: 5-10.

Parada L, Misteli T. (2002) Chromosome positioning in the interphase nucleus. Trends Cell Biol. 12(9): 425-432. Review.

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Parada LA, McQueen PG, Munson PJ, Misteli T. (2002) Conservation of relative chromosome positioning in normal and cancer cells. Curr Biol. 12(19): 1692-1697.

Park PC, De Boni U. (1998) A specific conformation of the territory of chromosome 17 locates ERBB-2 sequences to a DNase-hypersensitive domain at the nuclear periphery. Chromosoma. 107(2): 87-95.

Roix JJ, McQueen PG, Munson PJ, Parada LA, Misteli T. (2003) Spatial proximity of translocation-prone gene loci in human lymphomas. Nat Genet. 34(3): 287-291.

Shiomi H, Sugihara H, Kamitani S et al. (2003) Cytogenetic heterogeneity and progression of esophageal squamous cell carcinoma. Cancer Genet Cytogenet. 147(1): 50-61.

Sun HB. (2000) Size-dependent positioning of human chromosomes in interphase nuclei. Biophys J 79: 184-190.

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

Takehana T, Kunitomon K, Kono K. (2002) Status of c-erbB-2 in gastric adenocarcinoma: a comparative study of immunohistochemistry, fluorescence in situ hybridization and enzyme-linked immuno-sorbent assay. Int J Cancer 20; 98(6): 833-837.

Tanabe H, Habermann FA, Solovei I, Cremer M, Cremer T. (2002) Non-random radial arrangements of interphase chromosome territories: evolutionary considerations and functional implications. Mutat Res. 25;504(1-2): 37-45.

Visser AE, Aten JA. (1999) Chromosomes as well as chromosomal subdomains constitute distinct units in interphase nuclei. J Cell Sci. 112 (Pt 19): 3353-3360.

Volpi EV, Chevret E, Jones T et al. (2000) Large-scale chromatin organization of the major histocompatibility complex and other regions of human chromosome 6 and its response to interferon in interphase nuclei. J Cell Sci. 113 (Pt 9):1565-1576.

Williams RR. (2003) Transcription and the territory: the ins and outs of gene positioning. Trends Genet. 19(6): 298-302. Review.

Yamamoto T, Ikawa S, Akiyama T. (1986) Similarity of protein encoded by the human c-erbB-2 gene to epidermal growth factor receptor. Nature 319:230-234.

Yokota H, Singer MJ, van den Engh GJ, Trask BJ. (1997) Regional differences in the compaction of chromatin in human G0/G1 interphase nuclei. Chromosome Res. 5(3): 157-166.

Zink D, Cremer T, Saffrich R et al. (1998) Structure and dynamics of human interphase chromosome territories in vivo. Hum Genet. 102(2):241-251.

Lee- Análise de Concordância Kappa. (1995) Available at: http://www.lee.dante.br/pesquisa/kappa/index.html. Accessed January, 2005.

NCBI Entrez Gene. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi Accessed February, 2005.

Table 1. Results of location of the *CCND1* and *HER-2/neu* genes in the chromosome territories, amplification and protein overexpression in normal and tumor tissues of the esophagus.

Samples	*CCND1*					*HER-2/neu*				
	Position in CT			Amplification	Overexpression	Position in CT			Amplification	Overexpression
	I (%)	P (%)	Category	(G/C>2.0)	(>30%)	I (%)	P (%)	Category	(G/C>2.0)	(2+, 3+)
Normal mucosa										
MNE 01	65.1	34.9	I	0.9	35.4	63	37	I	1.0	0
MNE 02	24	75.8	P	1.0	25.2	68.5	31.4	I	0.9	0
MNE 03	76.5	23.4	I	0.9	25.4	74.2	25.8	I	0.9	0
MNE 04	36.2	63.7	P	0.9	20.4	67.3	32.6	I	0.9	0
MNE 05	19.7	80.3	P	0.9	12.2	59.7	40.2	I	1.0	0
($\bar{X}$ ± s)	44.30± 25.26	55.62± 25.24				66.54± 5.53	33.4± 5.51			
Esophageal carcinoma										
CE 01	3	97	P	**9.6**	20.4	22	77.9	P	1,0	1+
CE 02	2.9	96.9	P	**9.3**	22.2	20	79.9	P	1,0	0
CE 03	67.1	32.8	I	0.9	10.6	56	44	I	0,7	0
CE 04	77.2	22.7	I	0.9	11.8	11.2	88.7	P	**8,9**	0
CE 05	2.9	96.9	P	**9.4**	**36.6**	71.7	28.3	I	1,0	1+
CE 06	74	25.4	I	1.0	2.0	45.5	82.8	P	0,9	0
CE 07	74.6	25.4	I	**3.5**	22.3	12.2	87.7	P	**2,3**	**3+**
CE 08	25	75	P	0.9	**32.0**	71.7	28.2	I	**2,4**	0
CE 09	27.9	72.1	P	1.8	0	46.1	54	P	0,9	0
CE 10	38.8	61.3	P	**4.2**	**49.0**	35.4	64.6	P	0.9	**3+**
($\bar{X}$ ± s)	39.34± 31.55	60.55± 31.36				39.18± 22.78	63.61± 23.63			
P value		*0.5033*					*0.0203*			

Abbreviations: CT: chromosome territories; I: internal; P: peripheral; G/C: gene-to-centromere ratio.

Table 2. Results of location of the *CCND1* and *HER-2/neu* genes in the chromosome territories, amplification and protein overexpression in normal and tumor tissues of the stomach.

Samples	*CCND1*					*HER-2/neu*				
	Position in CT			Amplification	Overexpression	Position in CT			Amplification	Overexpression
	I (%)	P (%)	Category	(G/C>2.0)	(>20%)	I (%)	P (%)	Category	(G/C>2.0)	(2+, 3+)
Normal mucosa										
MNG 01	46	54	P	1.0	14.2	24.6	75.4	P	0,9	0
MNG 02	25	75	P	1.0	8.8	31	69	P	0,9	0
MNG 03	76.3	23.7	I	0.9	7.6	33	67	P	1.0	0
MNG 04	76.7	23.2	I	0.9	6.1	42	58	P	0.9	0
MNG 05	24.6	75.3	P	0.9	12.8	73.6	26.4	I	0.9	0
($\overline{X} \pm s$)	49.72± 25.93	50.24± 25.93				40.84± 19.34	59.16± 19.34			
Gastric cancer										
CG 01	12.2	87.7	P	1.5	**22.5**	36.6	63.4	P	0,9	0
CG 02	7.4	92.4	P	1.0	5.6	60	40	I	1,7	**2+**
CG 03	47.3	52.6	P	0.9	5.2	34.8	65.1	P	0,9	0
CG 04	1.6	98.3	P	0.9	**25.6**	24.6	75.2	P	0,9	0
CG 05	9	90.9	P	1.0	**20.2**	29.8	70.1	P	1,0	0
CG 06	15	85	P	1.0	**27.6**	19.3	80.5	P	1,0	0
CG 07	25.9	74	P	1.0	17.2	38.7	61.3	P	0.9	0
CG 08	17.9	82.1	P	0.9	**35.0**	25	74.9	P	1.0	0
CG 09	12.3	87.6	P	1.0	0.0	25.4	74.5	P	0.9	0
CG 10	13.2	86.7	P	0.9	12.0	37.8	62.2	P	0.9	0
($\overline{X} \pm s$)	16.18± 12.68	83.73± 12.67				33.20± 11.50	66.72± 11.44			
P value	*<0.0001*					*0.0017*				

Abbreviations: CT: chromosome territories; I: internal; P: peripheral; G/C: gene-to-centromere ratio.

Table 3. Relationship between gene location (peripheral or internal) of *CCND1* in chromosome 11 territory and amplification and overexpression, in esophageal carcinoma and gastric adenocarcinoma.

Samples/ Positions	Amplification (FISH)			Overexpression (IHC)		
	CCND1 (+)	*CCND1* (-)	Total	Cyclin D1 (+)	Cyclin D1 (-)	Total
Esophageal Carcinoma						
Peripheral	4 (40%)	2 (20%)	6 (60%)	3 (30%)	3 (30%)	6 (60%)
Internal	1 (10%)	3 (30%)	4 (40%)	0 (0%)	4 (40%)	4 (40%)
Total	5 (50%)	5(50%)	10 (100%)	3 (30%)	7 (70%)	10 (100%)
Kappa	*0.400**			*0.444**		
Gastric Cancer						
Peripheral	0 (0%)	10 (100%)	10 (100%)	5 (10%)	5 (90%)	10 (100%)
Internal	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Total	3 (30%)	7 (70%)	10 (100%)	5 (10%)	5 (90%)	10 (100%)
Kappa	*a*			*a*		

* Value of *Kappa* =0.40-0.59: moderated agreement
a Value of *Kappa* not estimated
FISH: Fluorescence *in situ* hybridization
IHC: immunohistochemical

Table 4. Relationship between gene location (peripheral or internal) of *HER-2/ neu* in chromosome 17 territory and amplification and overexpression, in esophageal carcinoma and gastric adenocarcinoma.

Samples/ Positions	Amplification (FISH)		Total	Overexpression (IHC)		Total
	HER-2/NEU (+)	*HER-2/NEU* (-)		Her-2/neu (+)	Her-2/neu (-)	
Esophageal Carcinoma						
Peripheral	2 (20%)	5 (50%)	7 (70%)	2 (20%)	5 (50%)	7 (70%)
Internal	1 (10%)	2 (20%)	3 (30%)	0 (0%)	3 (30%)	3 (30%)
Total	3 (30%)	7 (70%)	10 (100%)	2 (20%)	8 (80%)	10 (100%)
Kappa	*-0.034**			*0.194***		
Gastric cancer						
Peripheral	0 (0%)	9 (90%)	9(90%)	0 (0%)	9 (90%)	9 (90%)
Internal	0 (0%)	1 (10%)	1 (10%)	1 (10%)	0 (0%)	1 (10%)
Total	0 (0%)	10 (100%)	10 (100%)	1 (10%)	9 (90%)	10 (100%)
Kappa	*a*			*- 0.22**		

* Value of *Kappa* <0: no agreement
** Value of *Kappa*= 0-0.19: poor agreement
a Value of *Kappa* not estimated
FISH: Fluorescence *in situ* hybridization
IHC: immunohistochemical

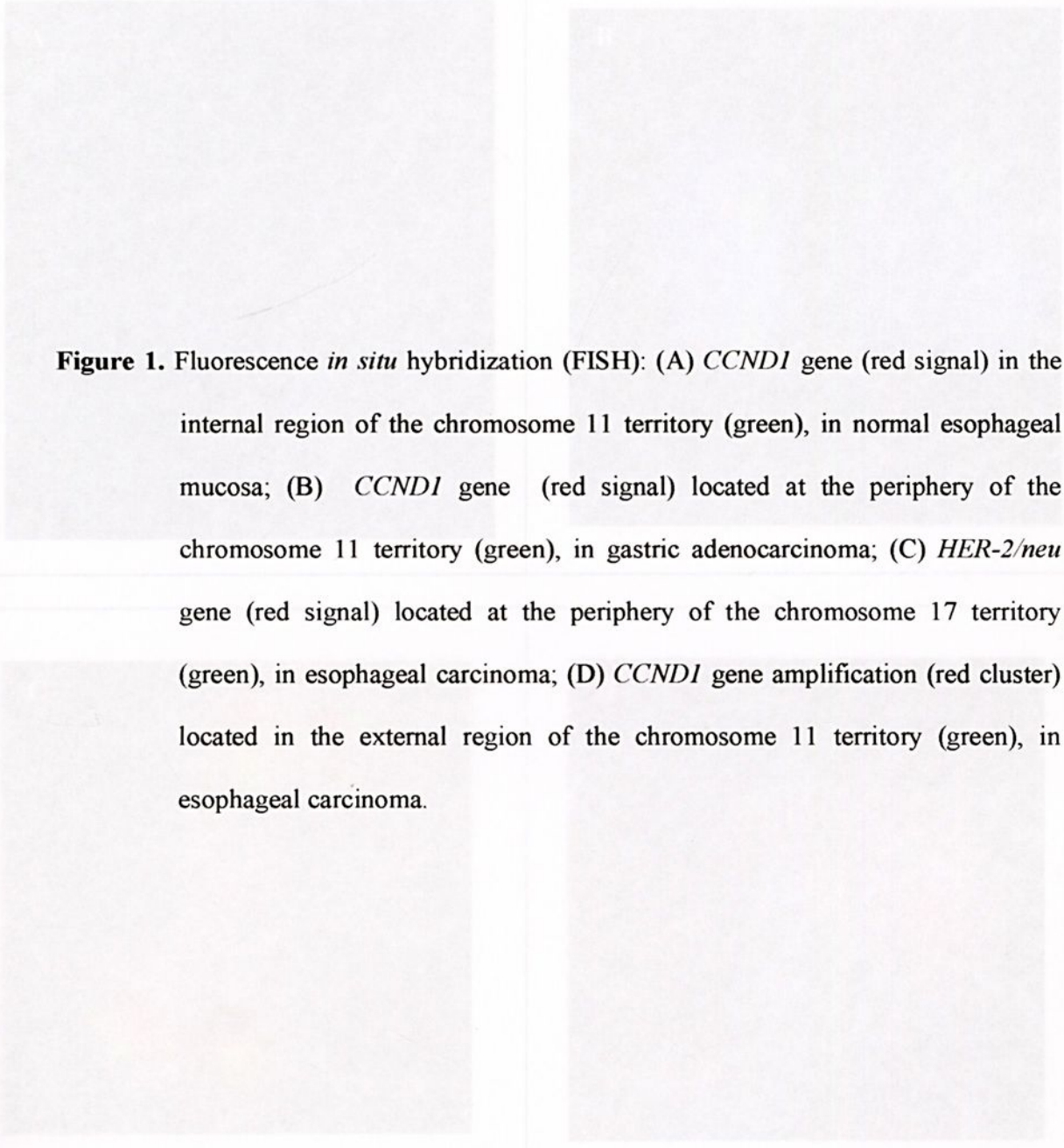
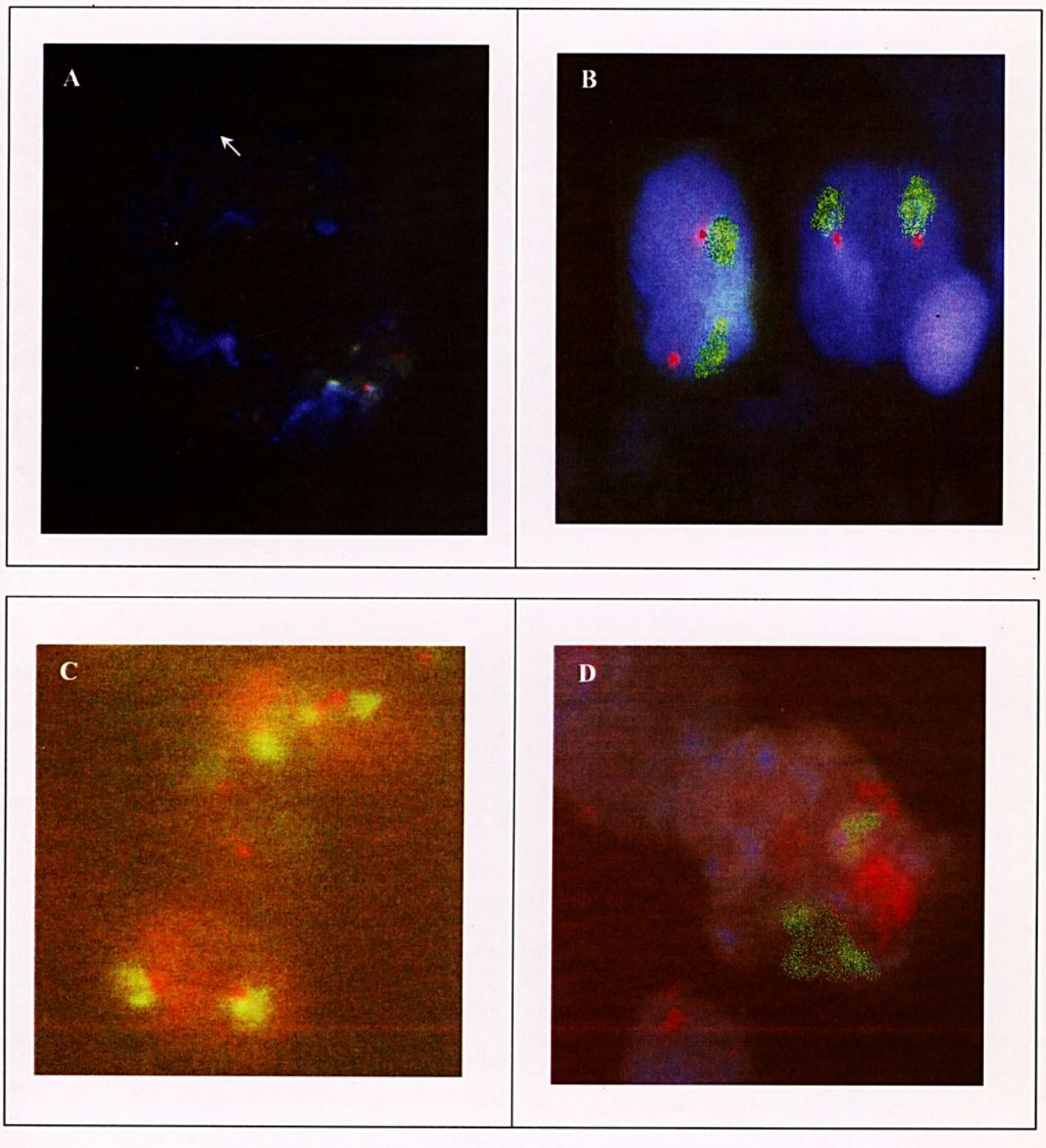
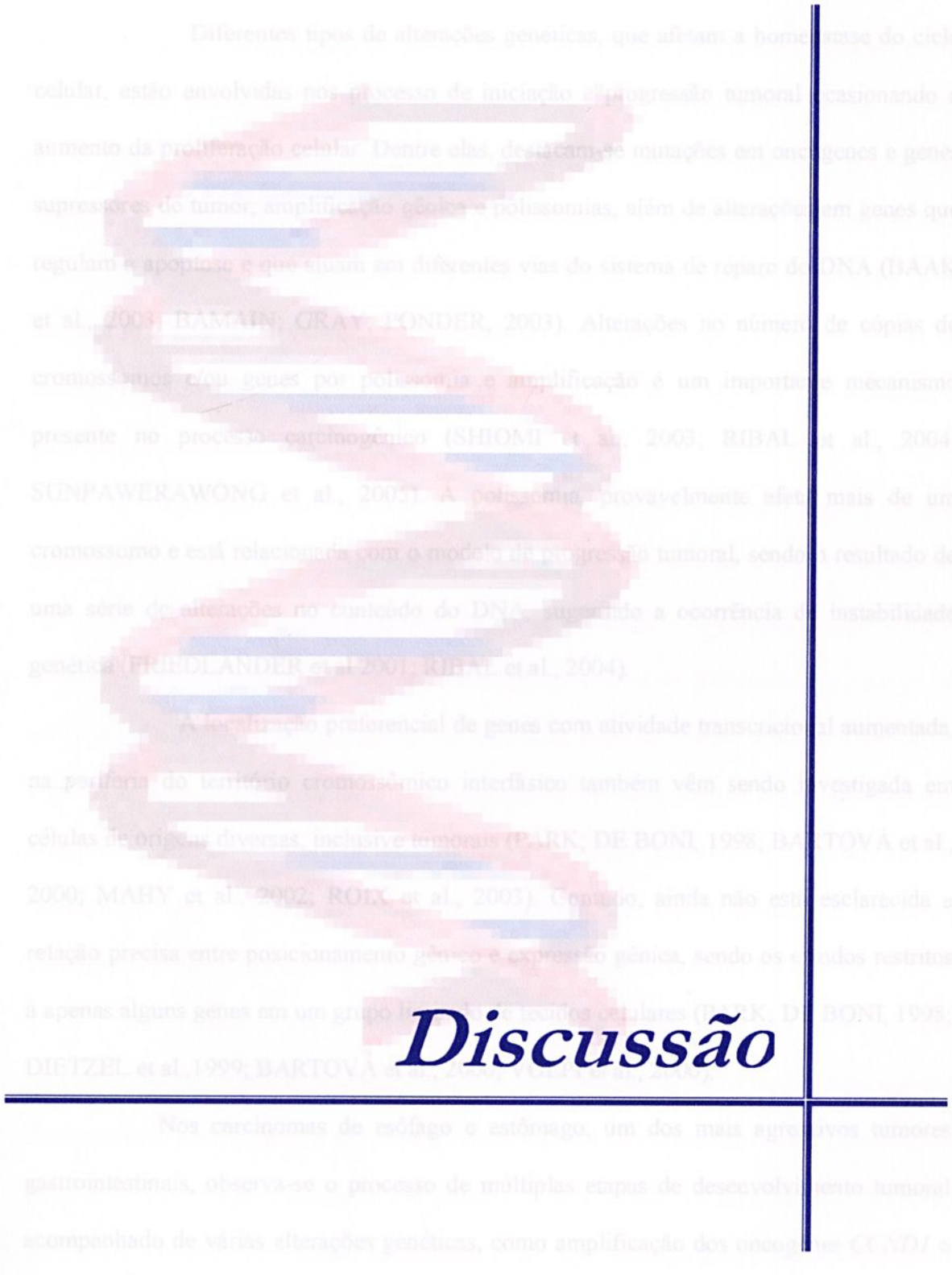


Figure 1. Fluorescence *in situ* hybridization (FISH): (A) *CCND1* gene (red signal) in the internal region of the chromosome 11 territory (green), in normal esophageal mucosa; (B) *CCND1* gene (red signal) located at the periphery of the chromosome 11 territory (green), in gastric adenocarcinoma; (C) *HER-2/neu* gene (red signal) located at the periphery of the chromosome 17 territory (green), in esophageal carcinoma; (D) *CCND1* gene amplification (red cluster) located in the external region of the chromosome 11 territory (green), in esophageal carcinoma.

cm 1 2 3 4 5 6 7 8 unesp 12 13 14 15 16 17 18 19


A
B
C
D



Discussão

Discussão

Diferentes tipos de alterações genéticas, que afetam a homeostase do ciclo celular, estão envolvidas nos processo de iniciação e progressão tumoral ocasionando o aumento da proliferação celular. Dentre elas, destacam-se mutações em oncogenes e genes supressores de tumor, amplificação gênica e polissomias, além de alterações em genes que regulam a apoptose e que atuam em diferentes vias do sistema de reparo do DNA (BAAK et al., 2003; BAMAIN; GRAY; PONDER, 2003). Alterações no número de cópias de cromossomos e/ou genes por polissomia e amplificação é um importante mecanismo presente no processo carcinogênico (SHIOMI et al., 2003; RIBAL et al., 2004; SUNPAWERAWONG et al., 2005). A polissomia, provavelmente afeta mais de um cromossomo e está relacionada com o modelo de progressão tumoral, sendo o resultado de uma série de alterações no conteúdo do DNA, sugerindo a ocorrência de instabilidade genética (FRIEDLANDER et al 2001; RIBAL et al., 2004).

A localização preferencial de genes com atividade transcricional aumentada, na periferia do território cromossômico interfásico também vêm sendo investigada em células de origens diversas, inclusive tumorais (PARK; DE BONI, 1998; BARTOVÁ et al., 2000; MAHY et al., 2002; ROIX et al., 2003). Contudo, ainda não está esclarecida a relação precisa entre posicionamento gênico e expressão gênica, sendo os estudos restritos à apenas alguns genes em um grupo limitado de tecidos celulares (PARK; DE BONI, 1998; DIETZEL et al.,1999; BARTOVÁ et al., 2000; VOLPI et al., 2000).

Nos carcinomas de esôfago e estômago, um dos mais agressivos tumores gastrointestinais, observa-se o processo de múltiplas etapas de desenvolvimento tumoral acompanhado de várias alterações genéticas, como amplificação dos oncogenes *CCND1* e

HER-2/neu (KAGAWA et al., 2000; SHIOMI et al., 2003; DAHLBERG et al., 2004; MANOEL-CAETANO et al., 2004). Assim como expressão aumentada de suas proteínas no tecido tumoral (AOYAGI et al., 2000; NAGASAWA et al., 2001; AKAMATSU et al., 2003; FEAKINS et al., 2003; DAHLBERG et al., 2004; GAO et al., 2004; SUNPAWERAWONG et al., 2005), mas nem sempre correlacionada com o nível de amplificação (TAKEHANA et al., 2002; DAHLBERG et al., 2004; SUNPAWERAWONG et al., 2005). Além do mais, apenas alguns estudos avaliaram a associação entre amplificação dos genes *CCND1* e *HER-2/neu* com expressão protéica em carcinomas de esôfago (DAHLBERG et al., 2004; SUNPAWERAWONG et al., 2005) e adenocarcinoma gástrico (TAKEHANA et al., 2002).

O presente estudo, avaliou conjuntamente os genes *CCND1* and *HER-2/neu* quanto ao ganho gênico (amplificação e/ou polissomia) pela técnica de FISH e quanto ao nível de expressão das proteínas por imuno-histoquímica. A associação entre esses parâmetros foi avaliada com o intuito de estabelecer o nível de correlação entre ganho no número de cópias gênicas e aumento da expressão protéica, como também a indicação como marcadores biológicos, se associados com fatores prognósticos e de sobrevivência. Este estudo também avaliou a localização desses oncogenes nos territórios cromossômicos interfásicos, assim como a associação com a ocorrência de amplificação e expressão elevada das proteínas nas mucosas normais e tecidos tumorais de esôfago e estômago.

O gene *CCND1* foi mais freqüentemente amplificado nos dois tipos de tumores avaliados e também apresentou níveis elevados de expressão da proteína, quando comparado ao gene *HER-2/neu*. Por outro lado, o ganho no número de cópias do oncogene *HER-2/neu* ocorreu principalmente por polissomia balanceada, em taxa elevada, que deve ter contribuído para uma expressão aumentada da proteína, especialmente naqueles casos

onde não foi observado amplificação gênica. Lal et al. (2003) evidenciaram que a polissomia 17 em câncer de mama teve uma participação importante na expressão elevada da proteína Her-2/neu nos casos sem amplificação e contribuiu para a imunomarcação positiva forte naqueles casos com amplificação. Do mesmo modo, em carcinomas de células escamosas de faringe e laringe as polissomias dos cromossomos 11 e 17 foram evidenciadas em freqüências elevadas (60-72%, respectivamente) (HARDISSON et al., 2004). Enquanto em adenocarcinoma gástrico, Gobbo-César et al. (2004) evidenciaram freqüências elevadas de polissomias dos cromossomos 3, 7, 8, 9 e 17.

Em carcinoma de esôfago a amplificação do gene *CCND1* foi mais freqüente (45%) do que a polissomia (25%), sendo o principal mecanismo envolvido no aumento da expressão da proteína. Tais achados reforçam as evidências de associação da amplificação desse gene com a carcinogênese do esôfago (JIN et al., 2004; MANOEL-CAETANO et al., 2004; SUNPAWERAWONG et al., 2005). Contudo, nos adenocarcinomas gástricos, tanto a amplificação do gene *CCND1* como a polissomia ocorreram em freqüências reduzidas (10-15%, respectivamente) corroborando a baixa freqüência (16,6%) relatada por Gao et al. (2004). Dessa forma, a amplificação do gene *CCND1* na tumorigênese do cancer gástrico não parece ser tão relevante como relatado por outros autores (YASOGAWA et al., 1998; TAKANO et al., 2000). Por outro lado, a proteína Ciclina D1 apresentou nível elevado de expressão em 37,5% dos casos de carcinoma de esôfago e em 35% dos casos de adenocarinoma gástrico, concordando com estudos anteriores, que têm relatado expressão aumentada dessa proteína em 26 a 71% dos tumores de esôfago (ARBER et al., 1999; NAGASAWA et al., 2001; SUNPAWERAWONG et al., 2005) e 25 a 50% dos tumores gástricos (AHN et al., 1998; ARBER et al., 1999; AOYAGI et al., 2000; GAO et al., 2004).

A associação entre amplificação ou polissomia do gene *CCND1* com a expressão aumentada da proteína Ciclina D1 foi muito baixa, sendo apenas observada uma concordância satisfatória em carcinoma de esôfago (25% dos casos) e adenocarcinoma gástrico (10% dos casos), apenas quando considerou-se o ganho gênico por amplificação. Poucos estudos têm avaliado a correlação entre esses dois parâmetros e os achados têm evidenciado associação de amplificação com expressão da proteína Ciclina D1 em 30-70% de carcinomas de esôfago (SHINOZAKI et al., 1996; JIN et al., 2004; SUNPAWERAWONG et al., 2005) e em 17% de adenocarcinoma gástrico (GAO et al., 2004). Também foi observado que nem todos os casos que apresentaram amplificação ou polissomia do gene *CCND1* tinham aumento da expressão da proteína, ou seja 14 casos de carcinoma de esôfago e três casos de adenocarcinoma gástrico tiveram ganho gênico, mas não tiveram os níveis da proteína elevados. De modo contrário, apenas um caso de carcinoma de esôfago e sete de adenocarcinoma gástrico apresentaram expressão elevada da proteína sem ganho gênico. Nesses últimos casos, outras vias devem promover a elevação da expressão da Ciclina D1, como alterações na síntese ou na estabilidade da proteína (NISHIMOTO et al., 2004), alterações na região promotora do gene *CCND1* ou regulação alterada dos fatores de transcrição, promovendo um aumento na produção de RNAm (GAO et al., 2004).

O gene *HER-2/neu* apresentou prevalência de ganho gênico por polissomia balanceada em alta freqüência, tanto em carcinoma de esôfago (32,5%) como em adenocarcinoma gástrico (15%), quando comparado com a amplificação gênica que foi observada em 7,5% e 5,0% dos casos, respectivamente. Sunpawerawong et al. (2005) também encontraram baixa freqüência de amplificação desse gene em tumores de esôfago (2%), em relação ao ganho gênico por polissomia (31%). Conseqüentemente, níveis

aumentados da proteína Her-2/neu foram encontrados em uma freqüência reduzida de casos, tanto em carcinoma de esôfago (12,5%) como nos adenocarcinomas gástricos (7,5%), mas conforme a freqüência relatada em estudos anteriores nesses dois tipos de tumores (HARDIWICK et al., 1997; DURSUN et al., 1999; ALLGAYER et al., 2000; ROSS et al., 2001; TAKEHANA et al., 2002; AKAMATSU et al., 2003; LAL et al., 2004).

A relação entre o ganho do gene *HER-2/neu* (amplificação e polissomia) com nível de expressão da proteína não está bem estabelecida. Nossos dados não mostraram concordância entre esses parâmetros em carcinoma de esôfago, porém foi evidenciado uma concordância satisfatória em adenocarcinoma gástrico. De modo geral, evidenciando que o ganho no número de cópias do gene *HER-2/neu* não é o principal mecanismo que leva à expressão aumentada da proteína. Mutações em ponto, translocações, alterações transcricionais e alterações em moléculas que controlam os sinalizadores também são outros eventos envolvidos no aumento da expressão da proteína Her-2/neu (ANTEAGA et al., 2002; PINIEUX et al., 2004).

Apesar das variações dos níveis de amplificação e expressão do gene *HER-2/neu* relatadas em diferentes tumores, ele parece ser um alvo interessante para terapia molecular. Takehana et al. (2002) sugeriram que os mecanismos de amplificação e expressão aumentada de *HER-2/neu* em adenocarcinoma gástrico parecem ser semelhantes aos encontrados em câncer de mama. Dalbergh et al. (2004) observaram inibição do crescimento da linhagem celular (OE19) de adenocarcinoma de esôfago após incubação com a droga Herceptin (trastuzumab). Portanto, os pacientes com tumores de esôfago e estômago que apresentam amplificação ou expressão aumentada da proteína Her-2/neu podem ser candidatos a terapia molecular com o anticorpo mococlonal trastuzumab (ALLGAYER et al., 2000; TAKEHANA et al., 2002; DAHLBERG et al., 2004).

cm 1 2 3 4 5 6 7 8 unesp 12 13 14 15 16 17 18 19

A análise combinada dos genes *CCND1* e *HER-2/neu* e das respectivas proteínas em tumores de esôfago e estômago não revelou associação na ocorrência de amplificação ou super expressão de ambos genes e proteínas nos tumores avaliados. Assim, sugerindo que não parece haver interação desses genes no processo carcinogênico do esôfago e estômago. Similarmente, Sunpawerawong et al (2005) também não encontraram associação entre os genes *CCND1, EGFR* and *HER-2/neu* quando avaliaram os níveis de amplificação e expressão conjuntamente em carcinoma de esôfago. Dessa forma, a falta de associação nos padrões de amplificação e expressão desses genes em tumores distintos, sugere mecanismos alternativos de ativação das vias de tumorigênese (SUNPAWERAWONG et al., 2005).

A indicação dos genes *CCND1* e *HER-2/neu* em situações de amplificação e/ou de expressão elevada, como marcadores de prognóstico e sobrevida em neoplasias do esôfago e estômago ainda é controversa na literatura (HARDWICK et al., 1997; AKAMATSU et al., 2003; SUNPAWERAWONG et al., 2005). No presente estudo não observamos associação entre os parâmetros demográficos (sexo e idade) e clinicopatológicos (consumo de álcool e de cigarro, graduação e tipo histológico, infecção pela bactéria *H. pilory* e sobrevivência) dos pacientes com tumores de esôfago e estômago com ganho dos genes *CCND1* e *HER-2/neu* (amplificação e polissomia) ou expressão aumentada das proteínas. Exceto que em pacientes com câncer gástrico observou-se uma associação entre consumo de álcool e ganho do gene *CCND1* por amplificação e polissomia, e também nível elevado da proteína Ciclina D1 em pacientes etilistas com carcinoma de esôfago. O consumo de álcool é um fator ambiental que parece atuar durante o processo de carcinogênese do esôfago e do estômago (WUNSCH-FILHO, 2002), devido a relação direta dos compostos N-nitrosos com o processo carcinogênico (DE VITA et al.,

1997; TAHARA et al., 2004). Assim evidenciando a interação gene-ambiente nesses tipos tumorais. Contudo, Kornfehl et al. (2002) não evidenciaram efeito do álcool sobre a expressão da Ciclina D1, como ocorreu com as proteínas p21, p18 e p19, em linhagem celular de carcinoma de células escamosas de cabeça e pescoço.

A atividade transcricional gênica têm sido correlacionada com o posicionamento dos genes nos repectivos territórios cromossômicos (TCs) ocupados nos núcleos interfásicos. Portanto, os genes *CCND1* e *HER-2/neu* constituem alvos interessantes para avaliação de posicionamento preferencial nos territórios dos cromossomos 11 e 17, utilizando como modelo células normais e tumorais cultivadas de esôfago e estômago, e sua associação com amplificação e expressão elevada das proteínas. De modo geral, os resultados apontaram uma localização preferencial dos genes *CCND1* e *HER-2/neu* na periferia dos TCs nas mucosas normais e tecidos tumorais de esôfago e estômago. No entanto, não foi observada uma associação precisa entre o posicionamento gênico com os padrões de amplificação e expressão das proteínas. Mas em carcinoma de esôfago a estatística *Kappa* mostrou uma concordância satisfatória a moderada entre localização do genes *CCND1* com amplificação e expressão protéica.

Esses resultados estão de acordo com alguns estudos prévios que evidenciaram posição periférica ou externa de genes em seus respectivos territórios cromossômicos, especialmente quando eles estavam sendo transcritos (PARK; DE BONI, 1998; DIETZEL et al.,1999; BARTOVÁ et al., 2000; VOLPI et al., 2000). Entretanto, essas observações ainda não são conclusivas, pois alguns outros estudos não evidenciaram uma associação da posição gênica com seu estado transcricional e genes ativos também foram observados no interior dos territórios cromossômicos. Por exemplo, Dietzel et al. (1999) observaram que o gene *ANT-2*, sujeito à inativação do cromossomo X, encontrava-

se na periferia do território do cromossomo X ativo, mas no interior do território do cromossomo X inativo, onde ele está silenciado. Volpi et al. (2000) demonstraram que os genes da região do complexo principal de histocompatibilidade (MHC) extendiam-se para fora do território do cromossomo 6, formando grandes alças quando as células eram estimuladas para transcrição desses genes. Similarmente, em células de câncer de mama, Park; De Boni et al. (1998) também encontraram protusões externas do gene *HER-2/neu* em relação ao território do cromossomo 17. Kurz et al. (1996), apesar de observar em cerca de 73-88% dos TCs os genes *DMD, MYH7 e HBB* na posição externa em células musculares, fibroblastos e células HeLa, essa localização era independente da atividade transcricional desses genes.

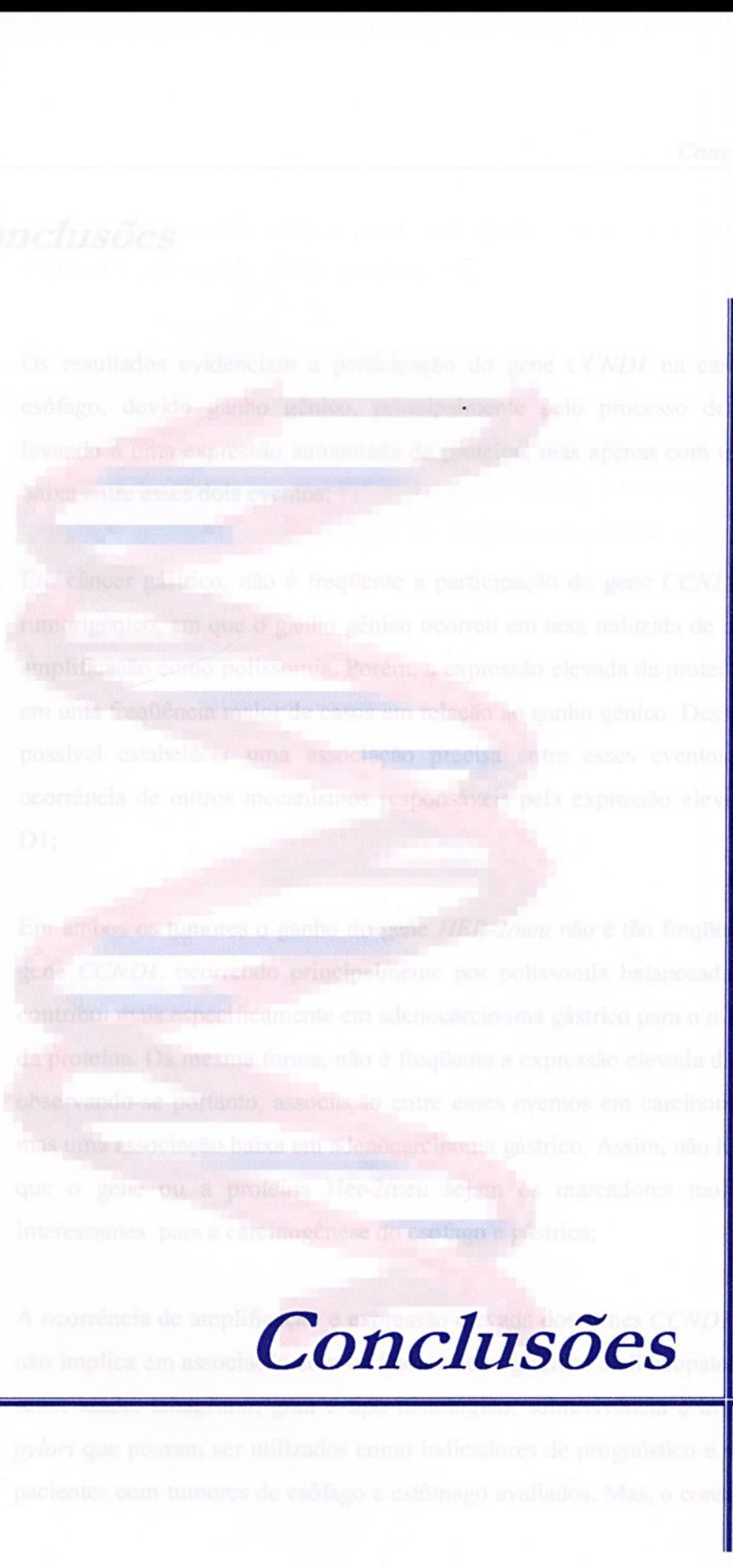
De modo contrário, Roix et al. (2003) constataram que o gene *CCND1* estava localizado mais freqüentemente na região interna do território do cromossomo 11, enquanto que outros genes, como o c-*MYC*, *BCL6* e *IGK* ocupavam preferencialmente a posição periférica dos respectivos territórios cromossômicos, em células de linfoma humano. Da mesma forma, Mahy et al. (2002), também observaram que o locus *WAGR* (11p13), que contém os genes *WT1*, *RCN*, *PAX6* e *PAXNEB* localizavam-se preferencialmente no interior do cromossomo 11 e quando ativados não resultava em mudanças de localização, ou seja, reposicionamento para a periferia do território. Portanto, outros aspectos devem ser considerados conjuntamente no direcionamento da localização dos genes nos TCs, como densidade gênica, taxa transcricional diferencial em regiões de banda G+ (pobre em genes) e banda R+ (rica em genes), estrutura da cromatina, posição do CT no núcleo, taxa e estabilidade de expressão e especificidade do tecido (VOLPI et al., 2000; KOSAK; GROUDINE, 2002).

cm 1 2 3 4 5 6 7 8 unesp 12 13 14 15 16 17 18 19

O modelo revisado de território cromossômico prediz a atividade gênica não restrita apenas a posição periférica dos TCs, devido a presença de uma rede de canais interconectados tornando os TCs permeáveis à proteínas e fatores de transcrição, sem zonas significantes de exclusão (PARADA; MISTELI, 2002).

Devido ao número limitado de estudos sobre a localização dos genes nos territórios cromossômicos interfásicos e a sua relação com atividade transcricional, ainda é pouco compreendido sobre o comportamento gênico durante o processo de transcrição, assim como a existência ou não de similaridade quanto a posição entre o grande número de genes, nos diferentes tipos de tecidos.

Conclusões



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a. Os resultados evidenciam a participação do gene *CCND1* na carcinogênese do esôfago, devido ganho gênico, principalmente pelo processo de amplificação, levando a uma expressão aumentada da proteína, mas apenas com uma associação baixa entre esses dois eventos;

b. Em câncer gástrico, não é freqüente a participação do gene *CCND1* no processo tumorigênico, em que o ganho gênico ocorreu em taxa reduzida de casos tanto por amplificação como polissomia. Porém, a expressão elevada da proteína é observada em uma freqüência maior de casos em relação ao ganho gênico. Dessa forma, não é possível estabelecer uma associação precisa entre esses eventos, sugerindo a ocorrência de outros mecanismos responsáveis pela expressão elevada da Ciclina D1;

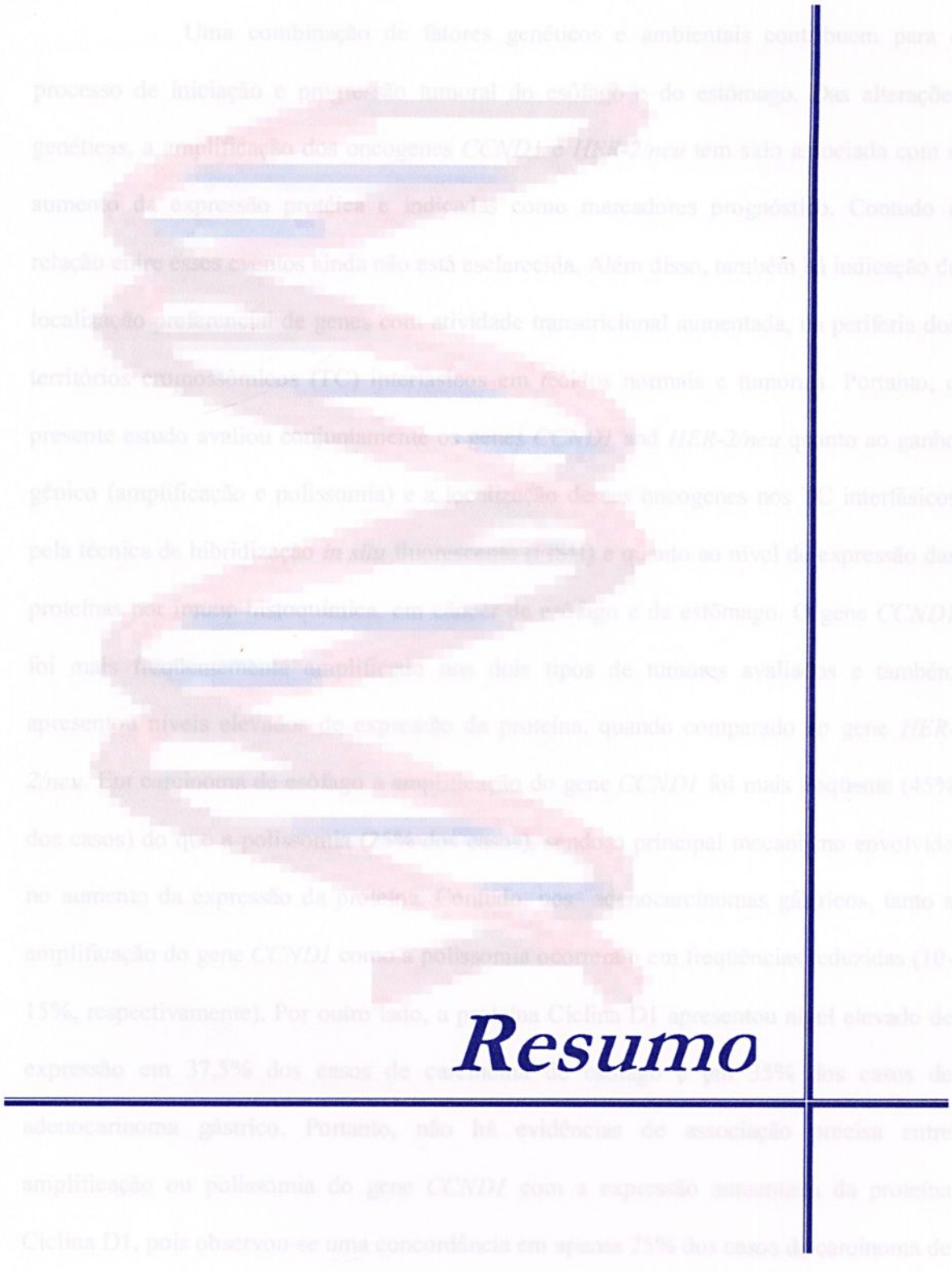
c. Em ambos os tumores o ganho do gene *HER-2/neu* não é tão freqüente como o do gene *CCND1*, ocorrendo principalmente por polissomia balanceada elevada, que contribui mais especificamente em adenocarcinoma gástrico para o nível aumentado da proteína. Da mesma forma, não é freqüente a expressão elevada da proteína, não observando-se portanto, associação entre esses eventos em carcinoma de esôfago, mas uma associação baixa em adenocarcinoma gástrico. Assim, não há indicação de que o gene ou a proteína Her-2/neu sejam os marcadores moleculares mais interessantes para a carcinogênese do esôfago e gástrica;

d. A ocorrência de amplificação e expressão elevada dos genes *CCND1* e *HER-2/neu* não implica em associação com os fatores demográficos e clinicopatológicos, como sexo, idade, tabagismo, grau e tipo histológico, sobrevivência e infecção pela *H. pylori* que possam ser utilizados como indicadores de prognóstico e sobrevida, nos pacientes com tumores de esôfago e estômago avaliados. Mas, o consumo de álcool

parece estar associado com os padrões de ganho gênico e expressão da proteína Ciclina D1, merecendo outras investigações;

e. Nossos resultados indicam uma localização preferencial dos oncogenes *CCND1* e *HER-2/neu* na periferia dos territórios dos cromossomos 11 e 17, mas de modo geral independente do tipo e histologia do tecido (mucosa normal ou tumoral de esôfago e estômago) e dos padrões de ganho gênico e expressão das proteínas, sugerindo a participação conjunta de outros mecanismos além da atividade transcricional.

Resumo



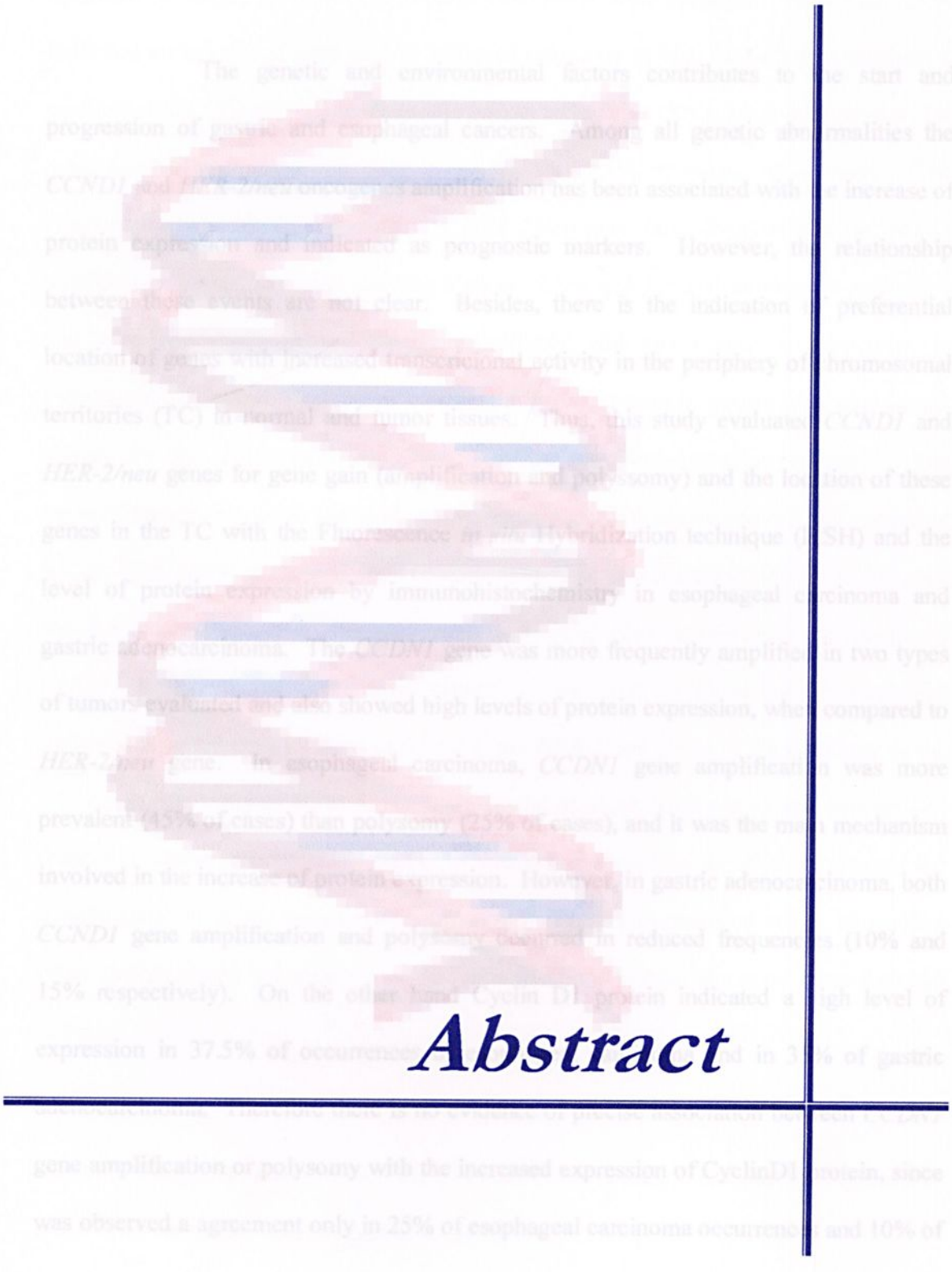
Resumo

Resumo

Uma combinação de fatores genéticos e ambientais contribuem para o processo de iniciação e progressão tumoral do esôfago e do estômago. Das alterações genéticas, a amplificação dos oncogenes *CCND1* e *HER-2/neu* tem sido associada com o aumento da expressão protéica e indicadas como marcadores prognóstico. Contudo a relação entre esses eventos ainda não está esclarecida. Além disso, também há indicação de localização preferencial de genes com atividade transcricional aumentada, na periferia dos territórios cromossômicos (TC) interfásicos em tecidos normais e tumorais. Portanto, o presente estudo avaliou conjuntamente os genes *CCND1* and *HER-2/neu* quanto ao ganho gênico (amplificação e polissomia) e a localização desses oncogenes nos TC interfásicos pela técnica de hibridização *in situ* fluorescente (FISH) e quanto ao nível de expressão das proteínas por imuno-histoquímica, em câncer de esôfago e de estômago. O gene *CCND1* foi mais freqüentemente amplificado nos dois tipos de tumores avaliados e também apresentou níveis elevados de expressão da proteína, quando comparado ao gene *HER-2/neu*. Em carcinoma de esôfago a amplificação do gene *CCND1* foi mais freqüente (45% dos casos) do que a polissomia (25% dos casos), sendo o principal mecanismo envolvido no aumento da expressão da proteína. Contudo, nos adenocarcinomas gástricos, tanto a amplificação do gene *CCND1* como a polissomia ocorreram em freqüências reduzidas (10-15%, respectivamente). Por outro lado, a proteína Ciclina D1 apresentou nível elevado de expressão em 37,5% dos casos de carcinoma de esôfago e em 35% dos casos de adenocarinoma gástrico. Portanto, não há evidências de associação precisa entre amplificação ou polissomia do gene *CCND1* com a expressão aumentada da proteína Ciclina D1, pois observou-se uma concordância em apenas 25% dos casos de carcinoma de

esôfago e 10% dos casos de adenocarcinoma gástrico quando considerou-se o ganho gênico por amplificação. Assim, outros mecanismos devem promover o aumento da expressão dessa proteína. O gene *HER-2/neu* apresentou prevalência de ganho gênico por polissomia balanceada em alta freqüência, tanto em carcinoma de esôfago (32,5%) como em adenocarcinoma gástrico (15%), quando comparado com a amplificação gênica que foi observada em apenas 7,5% e 5,0% dos casos, respectivamente. A relação entre o ganho do gene *HER-2/neu* (amplificação e polissomia) com nível de expressão da proteína, que apresentou-se elevada em 12,5% e 7,5% dos casos de câncer de esôfago e de estômago, respectivamente, não mostraram associação em carcinoma de esôfago. Porém foi evidenciado uma concordância satisfatória em adenocarcinoma gástrico, principalmente pela contribuição das polissomias no aumento da expressão protéica. Também não foi constatado uma associação entre os padrões de ganho gênico e de expressão das proteínas com parâmetros como sexo, idade, tabagismo, grau histológico e infecção pela bactéria *Helicobacter pylori*, mas o etilismo foi associado com ganho gênico ou expressão aumentada do gene *CCND1* em câncer de esôfago e gástrico. O estudo dos territórios cromossômicos apontaram uma localização preferencial dos genes *CCND1* e *HER-2/neu* na periferia dos TC (freqüência média de 60-83%) nos núcleos dos tecidos tumorais de esôfago e de estômago e respectivas mucosas normais. No entanto, não observou-se uma associação entre o posicionamento gênico no TC com os padrões de amplificação e expressão das proteínas, sugerindo-se que outros fatores devem atuar conjuntamente na localização dos genes, além da atividade gênica transcricional.

Palavras chaves: *CCND1*, *HER-2/neu*, FISH, imuno-histoquímica, território cromossômico, câncer de esôfago e câncer de estômago.



Abstract

Abstract

The genetic and environmental factors contributes to the start and progression of gastric and esophageal cancers. Among all genetic abnormalities the *CCND1* and *HER-2/neu* oncogenes amplification has been associated with the increase of protein expression and indicated as prognostic markers. However, the relationship between these events are not clear. Besides, there is the indication of preferential location of genes with increased transcricional activity in the periphery of chromosomal territories (TC) in normal and tumor tissues. Thus, this study evaluated *CCND1* and *HER-2/neu* genes for gene gain (amplification and polyssomy) and the location of these genes in the TC with the Fluorescence *in situ* Hybridization technique (FISH) and the level of protein expression by immunohistochemistry in esophageal carcinoma and gastric adenocarcinoma. The *CCDN1* gene was more frequently amplified in two types of tumors evaluated and also showed high levels of protein expression, when compared to *HER-2/neu* gene. In esophageal carcinoma, *CCDN1* gene amplification was more prevalent (45% of cases) than polysomy (25% of cases), and it was the main mechanism involved in the increase of protein expression. However, in gastric adenocarcinoma, both *CCND1* gene amplification and polysomy occurred in reduced frequencies (10% and 15% respectively). On the other hand Cyclin D1 protein indicated a high level of expression in 37.5% of occurrences of esophageal carcinoma and in 35% of gastric adenocarcinoma. Therefore there is no evidence of precise association between *CCDN1* gene amplification or polysomy with the increased expression of CyclinD1 protein, since was observed a agreement only in 25% of esophageal carcinoma occurrences and 10% of

gastric adenocarcinoma ones, when considered the genetic gain by amplification. Other mechanisms might promote this protein expression level increase. *HER-2/neu* gene indicated prevalence of gene gain by balanced polysomy in high frequency in esophageal carcinoma (32.5%) as well as in gastric adenocarcinoma (15%), when compared to the gene amplification observed only in 12.5% and 7.5% of cases, respectively. The relationship between the gene gain of *HER-2/neu* gene (amplification and polysomy) with protein expression level that was high in 12.5% and 7.5% of esophageal carcinoma and gastric adenocarcinoma cases, respectively, did not indicated association in esophageal carcinoma. However it indicated a fair agreement in gastric adenocarcinoma, mainly by the polysomies contribution in the protein expression increase. The association between gene gain and protein expression with regards to sex, age, smoking habits, histological grade and *Helicobacter pylori* bacteria infection was not evidenced, but the alcohol consume was associated with gene gain or increased expression of *CCDN1* in esophageal and gastric cancers. The study of chromosomes territories indicated a preferential location of *CCDN1* and *HER-2/neu* genes in the periphery of the TC (mean frequency of 60 to 83%) in the nuclei of esophageal and gastric cancers and respective normal mucosas. However, it was not observed an association between the genetic position in TC with the amplification and protein expression status, suggesting other factors might act together in the gene location, besides the gene transcriptional activity.

Referências Bibliográficas

Keywords: *CCDN1*, *HER*-2/neu, FISH, immunohistochemistry, chromosome territories, esophageal carcinoma and gastric adenocarcinoma.

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Referências Bibliográficas

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AHN, M.J. et al. Expression of cyclin D1 and cyclin E in human gastric carcinoma and its clinicopathologic significance. *J. Korean Med. Sci.*; v.13, n.5, p.513-518, 1998.

AKAMATSU, M. et al. c-erbb-2 oncoprotein expression related to chemoradioresistance in esophageal squamous cell carcinoma. *Int. J. Radiat. Oncol. Biol. Phys.*; v.57, n.5, p.1323-1327, 2003.

ALLGAYER, H. et al. C-erbB-2 is of independent prognostic relevance in gastric cancer and is associated with the expression of tumor-associated protease systems. *J. Clin. Oncol.*; v.18. n.11, p.2201-2209, 2000.

AOYAGI, K. et al. Immunohistochemical study on the expression of cyclin D1 and E in gastric cancer. *Kurume Med. J.* v.47n.3, p.199-203, 2000.

ARBER N. et al. Overexpression of Cyclin D1 occurs in both squamous carcinomas and adenocarcinomas of the esophagus and in adenocarcinomas of the stomach. *Hum. Pathol.*; v.30, n.9, p.1087-1092, 1999.

ARTEAGA, C.L. Epidermal growth factor receptor dependence in human tumors: more than just expression? *Oncologist* v.7, n.4, p.31-39, 2002.

BAAK, J.P.A. et al. Genomics and proteomics in cancer. *European J. Cancer* v.39, p.1199-1215, 2003.

BALMAIN, A.; GRAY, J.; PONDER, B. The genetics and genomics of cancer. *Nature Genet (supplement)* v.33, p.238-244, 2003.

BAHL, R. et al. Novel polymorphism in p21(waf1/cip1) cyclin dependent kinase inhibitor gene: association with human esophageal cancer. *Oncogene* v.20, n.19(3), p.323-328, 2000.

BARTOVA, E. et al. Nuclear topography of the c-myc gene in human leukemic cells. *Gene*, v.22, n.244(1-2), p.1-11, 2000.

BAST ROBERT, C. et al. *Cancer Medicine*. BC Decker Inc. 5th edition, Ontario, 2000.

BECKER, K.F.; KELLER, G.; HOEFLER, H. The use of molecular biology in diagnosis and prognosis of gastric cancer. *Surg. Oncol.* v.9, p.5-11, 2000.

BRIEN, T.P. et al. HER-2/neu gene amplification by FISH predicts poor survival in Barret's esophagus-associated adenocarcinoma. *Hum. Pathol.*, v.31, p.35-39, 2000.

CEZAR A.C. et al. Aneuploidies, deletion, and overexpression of TP53 gene in intestinal metaplasia of patients without gastric cancer. *Cancer Genet. Cytogenet*. v.153, n.2, p.127-132, 2004.

cm 1 2 3 4 5 6 7 8 unesp 12 13 14 15 16 17 18 19

CHEVRET, E.; VOLPI, E.V.; SHEER, D. Mini review: form and function in the human interphase chromosome. *Cytogenet. Cell Genet.* v.90, n.1-2, p.13-21, 2000.

CORREA, P. The biological model of gastric carcinogenesis. *IARC Sci Publ.* v.157, p.301-310, 2004.

CORREA, P.; MILLER, M.J.S. Carcinogenesis, apoptosis and cell proliferation. *Br. Med. Bull.*, v.54, p.151-162, 1998.

COTRAN, R.S.; ROBBINS, S.L.; KUMAR, V. *Patologia Estrutural e Funcional*, 4ª ed., Rio de Janeiro: Guanabara, 130p., 1991.

CREMER, T. et al. Role of chromosome territories in the functional compartmentalization of the cell nucleus. *Cold. Spring Har.b Sym.p Quant. Biol.* v.58, p.777-792, 1993

CREMER, M. et al. Inheritance of gene density-related higher order chromatin arrangements in normal and tumor cell nuclei. *J. Cell Biol.* v.1, n.162(5), p.809-820, 2003.

CREMER, T. et al. Higher order chromatin architecture in the cell nucleus: on the way from structure to function. *Biol. Cell.* v.96, n.8, p.555-567, 2004.

CROFT, J.A. et al. Differences in the localization and morphology of chromosomes in the human nucleus. *J. Cell Biol.* v.14, n.145(6), p.1119-1131, 1999.

DAHLBERG, P.S. et al ERBB2 amplifications in esophageal adenocarcinoma. *Ann. Thorac. Surg.* v.78, n.(5), p.1790-800, 2004.

DE-VITA, V.J.R.; HELLMAN, S.; ROSENBERG, S.A. Cancer - *Principles and Practice of Oncology*, 5th ed. v.1: Philadelphia, New York: Lippincott-Raven, p.1154, 1997.

DURSUN A.et al. Expression of c-erbB-2 oncoprotein in gastric carcinoma: correlation with histopathologic characteristics and analysis of Ki-67. *Pathol. Oncol. Res.*; v.5, n.2, p.104-106, 1999.

DIETZEL, S.; SCHIEBEL, K.; LITTLE, G. The 3D positioning of ANT2 and ANT3 genes within female X chromosome territories correlates with gene activity. *Exp. Cell Res.* v.1, n.252(2), p.363-375, 1999.

EDELMANN, P. et al. Morphology and dynamics of chromosome territories in living cells. *Biochim. Biophys. Acta.* v.1551, n.1, 2001.

EWEN, M.E.; LAMB, J. The activities of cyclin D1 that drive tumorigenesis. *Trends Mol. Med.*, v.10, n.4, p.158-162, 2004.

FALK, M. et al. Topography of genetic elements of X-chromosome relative to the cell nucleus and to the chromosome X territory determined for human lymphocytes. *Gene*, v.12, n.292(1-2), p.13-24, 2002.

FEAKINS, R.M. et al. Abnormal expression of pRb, p16, and cyclin D1 in gastric adenocarcinoma and its lymph node metastases: relationship with pathological features and survival. *Hum. Pathol.*; v.34, n.12, p.1276-1282, 2003.

FRIEDLANDER, P.L. Genomic instability in head and neck câncer patients. *Head Neck* v.23, n.8, p. 683-691, 2001.

GAO, P. et al. Alteration of cyclin D1 in gastric carcinoma and its clinicopathologic significance. *World J. Gastroenterol.*; v.15, n.10(20), p.2936-2939, 2004.

GUILLEM, P. et al. Mucin gene expression and cell differentiation in human normal, premalignant and malignant esophagus. *Int. J. Cancer*. v.15, n.88(6), p.856-861, 2000.

HARDISSON, D. et al. Numerical aberrations of chromosomes 8, 9, 11, and 17 in squamous cell carcinoma of the pharynx and larynx: a fluorescence in situ hybridization and DNA flow cytometric analysis of 50 cases. *Oral Oncol.*; v.40, n.4, p.409-417, 2004.

HARDWICK, R.H. et al. Immunohistochemical detection of p53 and c-erbB-2 in oesophageal carcinoma; no correlation with prognosis. *Eur. J. Surg. Oncol.*; v.23, n.1, p.30-35, 1997.

HEAVEY, P.M.; ROWLAND, I.R. Microbial-gut interactions in health and disease. Gastrointestinal cancer. *Best. Pract. Res. Clin. Gastroenterol.*, v.18, n.2, p.323-336, 2004.

HENDZEL, M.J. et al. Compartmentalization of regulatory proteins in the cell nucleus. *J. Steroid Biochem. Mol. Biol.* v.76, n.1-5, p.9-21, 2001.

HIRATA, Y. et al. Helicobacter pylori activates the cyclin D1 gene through mitogen activated protein kinase pathway in gastric cancer cells. *Infect Immun.*, v.69, n.6, p.3965-3971, 2001.

HOLCOMBE, C. *Helicobacter pylori*: the African enigma. *Gut.*, v.33, p.429-431, 1992.

HOHENBERGER, P.; GRETSCHEL, S. Gastric cancer. *Lancet*; v.362, n.9380, p.305-315, 2003.

HUANG, X,.et al. High-resolution mapping of the 11q13 amplicon and identification of a gene, TAOS1, that is amplified and overexpressed in oral cancer cells. *PNAS* v.99, p.11369-11374, 2002.

IARC – Working Group on the Evaluation of Carcinogenic Risks to Humans. Schistosomes, liver flukes and Helicobacter pylori. IARC Working Group on the

Evaluation of Carcinogenic Risks to Humans. Lyon, 7-11 June 1994. IARC *Monogr. Eval. Carcinog. Risks Hum.*, v. 61, p.1-241, 1994.

INCA – INSTITUTO NACIONAL DO CÂNCER, MINISTÉRIO DA SAÚDE. *Estimativa da incidência e mortalidade por câncer no Brasil.* Rio de Janeiro, 2005. Disponível em www.inca.org.br

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

JIN, Y. et al. Cytogenetic and fluorescence in situ hybridization characterization of clonal chromosomal aberrations and CCND1 amplification in esophageal carcinomas. *Cancer Genet Cytogenet.*, v.148, n.1, p.21-28, 2004.

KAGAWA Y. et al. Significance of the expression of $p27^{KipI}$ in esophageal squamous cell carcinomas. *Dis. Esophagus.*, v.13, n.3, p.179-184, 2000.

KOO, S.H. et al. Genetic alterations of gastric cancer: comparative genomic hybridization and fluorescence in situ hybridization studies. *Cancer Genetic Cytogenetic*, v.117, p.97-103, 2000.

KORNFEHL, J. et al. Ethanol decreases negative cell-cycle-regulating proteins in a and neck squamous cell carcinoma cell line. *Acta Otolaryngol.* v.122, n.3, p.338-342, 2002.

KOSAK ST, GROUDINE M. The undiscovered country: chromosome territories and the organization of transcription. *Dev. Cell.* v.2, n.6, p.690-692, 2002.

KURZ, A. et al. Active and inactive genes localize preferentially in the periphery of chromosome territories. *J. Cell Biol.* v.135, n.5, p.1195-1205, 1996.

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.

LAL, P. et al. Impact of Polysomy 17 on HER-2/neu immunohistochemistry in breast carcinomas without HER-/neu gene amplification. *JMD* v.5, n.3, p.155-159, 2003.

LEHRBACH, D.M.; NITA, M.E.; CECCONELLO, I. Molecular aspects of esophageal squamous cell carcinoma carcinogenesis. *Arq. Gastroenterol.* v.40, n.4, p.256-261, 2004.

LEAR-KAUL, K.C. et al. Her-2/neu status in breast cancer metastases to the central nervous system. *Arch. Pathol. Lab. Med.* v.127, p.1451-1457, 2003.

MAHY, N.L. et al. Spatial organization of active and inactive genes and noncoding DNA within chromosome territories. *J. Cell Biol.* v.13, n.157(4), p.579-89, 2002.

unesp

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

METZGER, R. et al. Molecular biology of esophageal cancer. *Onkologie* v.27, n.2, p.200-206, 2004.

MYIAMOTO, R. et al. Prognostic significance of cyclin D1 amplification and overexpression in oral squamous cell carcinomas. *Oral Oncol.* v.39, n.(6), p.610-618, 2003

MILLER, C.T. et al. Gene amplification in esophageal adenocarcinomas and Barret´s with high-grade dysplasia. *Clin. Cancer Res.* v.9, p.4819-4825, 2003.

MORENO-BUENO, G. et al. Cyclin D1 gene (CCND1) mutations in endometrial cancer. *Oncogene*, v.22, p.6115-6118, 2003.

MOSS, S.F. The carcinogenic effect of H. pylori on the gastric epithelial cell. *J. Phys. Pharmacol.* v.50, p.847-856, 1999.

NADAL, A.; CARDESA, A. Molecular biology of laryngeal squamous cell carcinoma. *Virchows Arch.* v. 442, n.1, p. 1-7, 2003.

NAGASAWA, S. et al. Cyclin D1 overexpression as a prognostic factor in patients with esophageal carcinoma. *J. Surg. Oncol.* v.78, n.3, p.208-214, 2001.

NESSLING, M. et al. Mapping of chromosomal imbalances in gastric adenocarcinoma revealed amplified protooncogenes MYCN, MET, WNT2, and ERBB2. Genes *Chromosomes Cancer*, v.23, p.307-316, 1998.

NISHIMOTO, I.N. et al. Cyclin D1 gene polymorphism as a risk factor for squamous cell carcinoma of the upper aerodigestive system in non-alcoholics. *Oral Oncology*; v.40, p.604-610, 2004.

NITA, M.E. et al. p21Waf1/Cip1 expression is a prognostic marker in curatively resected esophageal squamous cell carcinoma, but not p27Kip1, p53, or Rb. *Ann. Surg. Oncol.*, v.6, n.5, p.481-488, 1999.

NOGUCHI, T. et al. Chromosomal imbalance in esophageal squamous cell carcinoma: 3q gain correlates with tumor progression but not prognostic significance. *Oncol. Rep.* v.10, n.5, p.1393-1400, 2003.

ORMANDY, C.J. et al. Cyclin D1, EMS and 11q13 amplification in breast cancer. Breast *Cancer Res. Treat.*, v.78, n.3, p.323-335, 2003.

OZAWA, S.; KITAGAWA, Y.; KITAJIMA, M. Molecular alterations in esophageal cancer. *Nippon Geka Gakkai Zasshi* v.103, p.457-462, 2002.

PARADA, L.; MISTELI T. Chromosome positioning in the interphase nucleus. *Trends Cell Biol.*, v.12, n.9, p.425-432, 2002.

PARADA, L. A.; SOTIRIOU, S.; MISTELI, T. et al. Spatial genome organization. *Exp. Cell Res.*, v.15, n.296(1), p.64-70, 2004.

PARK, P.C.; DE BONI, U. A specific conformation of the territory of chromosome 17 locates ERBB-2 sequences to a DNase-hypersensitive domain at the nuclear periphery. *Chromosoma*. v.107, n.2, p.87-95, 1998.

PARKIN, D.M. Global cancer statistics in the year. *Lancet Oncol.* v.2, n.9, p.533-543. Erratum in: Lancet Oncol 2001 Oct;2(10):596, 2000.

PENG, D.F. et al. Alterations of chromosomal copy number during progression of diffuse-type gastric carcinomas: metaphase- and array-based comparative genomic hybridization analyses of multiple samples from individual tumours. *J. Pathol.* v.201, n.3, p.439-450, 2003.

PINIEUX, G. et al. Confrontation of immunohistochemistry and fluorescent in situ hybridization for the assessment of HER-2/neu (c-erbb-2) status in urothelial carcinoma. *Virchows Arch.*; v.444, n.5, p.415-419. Epub 2004 Mar 17, 2004.

POLAT, A. et al. Expression of cell-cycle related proteins in Helicobacter pylori gastritis and association with gastric carcinoma. *Neoplasma* v.49, n.2, p.95-100, 2002.

PUTZ, A. et al. TP53 mutation pattern of esophageal squamous cell carcinomas in a high risk area (Southern Brazil): role of life style factors. *Int. J. Cancer*. v.1, n.98(1), p.99-105, 2002.

RAGOCZY, T. et al. A genetic analysis of chromosome territory looping: diverse roles for distal regulatory elements.*Chromosome Res.* v.11, n.5, p.513-25, 2003.

RIBAL, M.J. et al. Chromosomal high-polysomies predict tumour progression in T1 transitional cell carcinoma of the bladder. *Eur. Urol.*, v.45, n.5, p.593-599, 2004.

ROIX, J.J. et al. Spatial proximity of translocation-prone gene loci in human lymphomas. *Nat Genet*. v.34, n.3, p.287-291, 2003.

ROSS, J.S.; MCKENNA, B.J. The HER-2/neu oncogenes in tumors of the gastrointestinal tract. *Cancer Invest.*; v.1, p.554-568, 2001.

SCHURING, E. The involvement of the chromosome 11q13 region in human malignancies: cyclin D1 and EMS1 are two new candidate oncogenes – a review. *Gene*; v.159, p.83-96. 1995.

SEGAL, I. et al. Gastric cancer in sub-Saharan Africa. *Eur. J. Cancer Prev.* v.10, n.6, p.479-82, 2001.

SEPULVEDA, A.R.; COELHO, L.G. et al. Helicobacter pylori and gastric malignancies. *Helicobacter* v.7, n.1, p.42, 2002.

SHINOZAKI, H. et al. Cyclin D1 amplification as a new predictive classification for squamous cell carcinoma of the esophagus, adding gene information. *Clin. Cancer Res.* v.2, p.1155-1161, 1996.

SHIOMI H et al. Cytogenetic heterogeneity and progression of esophageal squamous cell carcinoma. *Cancer Genet. Cytogenet.* v.147, n.1, p.50-61, 2003.

SONG, D.H. et al. Gastrin-induced gastric adenocarcinoma growth is mediated through cyclin D1. *Am. J. Physiol. Gastrointest. Liver Physiol.*, v.285, n.1, p.G217-222, 2003.

STAMOULI, M.I. et al. Application of multiplex fluorescence in situ hybridization in the cytogenetic analysis of primary gastric carcinoma. *Cancer Genet. Cytogenet.* v.135, n.1, p.23-27, 2002.

SUNPAWERAVONG, 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.

TAHARA E. Genetic pathways of two types of gastric cancer. *IARC Sci Publ*; v.157, p.327-349, 2004.

TAKANO, Y. et al. Cyclin D2 overexpression and lack of p27 correlate positively and cyclin E inversely with a poor prognosis in gastric cancer cases. *Am. J. Pathol.*; v.156, p.585-594, 2000.

TAKEHANA, T.; KUNITOMON, K.; KONO, K. Status of c-erbB-2 in gastric adenocarcinoma: a comparative study of immunohistochemistry, fluorescence in situ hybridization and enzyme-linked immuno-sorbent assay. *Int. J. Cancer*, v.20, n.6, p.833-837, 2002.

TANABE, H et al. Non-random radial arrangements of interphase chromosome territories: evolutionary considerations and functional implications. *Mutat. Res.* v.25, n. 504(1-2), p.37-45, 2002.

VARIS, A. et al. Targets of gene amplification and overexpression at 17q in gastric cancer. *Cancer Res.* v.62, p.2625-2629, 2002.

VERSHURE, P.J. et al. Spatial relationship between transcription sites and chromosome territories. *J. Cell Biol.*, v.147, p.13-24, 1999.

VIDGREN, V. et al. Concomitant gastrin and ERBB2 gene amplifications at 17q12-q21 in the intestinal type of gastric cancer. *Genes Chromosomes Cancer* v.24, p.24-29, 1999.

unesp

VISSER, A.E.; ATEN, J.A. Chromosomes as well as chromosomal subdomains constitute distinct units in interphase nuclei. *J. Cell Sci.* v.112, p.3353-3360, 1999.

VOLPI, E.V. et al. Large-scale chromatin organization of the major histocompatibility complex and other regions of human chromosome 6 and its response to interferon in interphase nuclei. *J. Cell Sci.* v.113, p.1565-1576, 2000.

WILLIAMS, R.R. Transcription and the territory: the ins and outs of gene positioning. *Trends Genet.* v.19, n.6, p.298-302, 2003.

WORLD CANCER RESEARCH FUND. Food, nutrition and the prevention of cancer: a global perspective. Washington, DC: *World Cancer Research Fund*, 1997.

WÜNSCH-FILHO V. The epidemiology of oral and pharynx cancer in Brazil. Oral *Oncology* v.38, p.737-746, 2002.

YAMAMOTO, T.; IKAWA, S.; AKIYAMA, T. Similarity of protein encoded by the human c-erbB-2 gene to epidermal growth factor receptor. *Nature* v.319, p.230-234, 1986.

YASOGAWA, Y. et al. The 5D4 antibody (anti-cyclin D1/D2) related antigen: Cytoplasmatic staining is correlated to the progression of gastric cancer. *Pathol. Int.*; v.48, p.717-722, 1998.

ZIRBEL, R.M. et al. Evidence for nuclear compartment of transcription and splicing located at chromosome domaisn boundaries. *Chromosome Res.*, v.1, p.93-106, 1993.

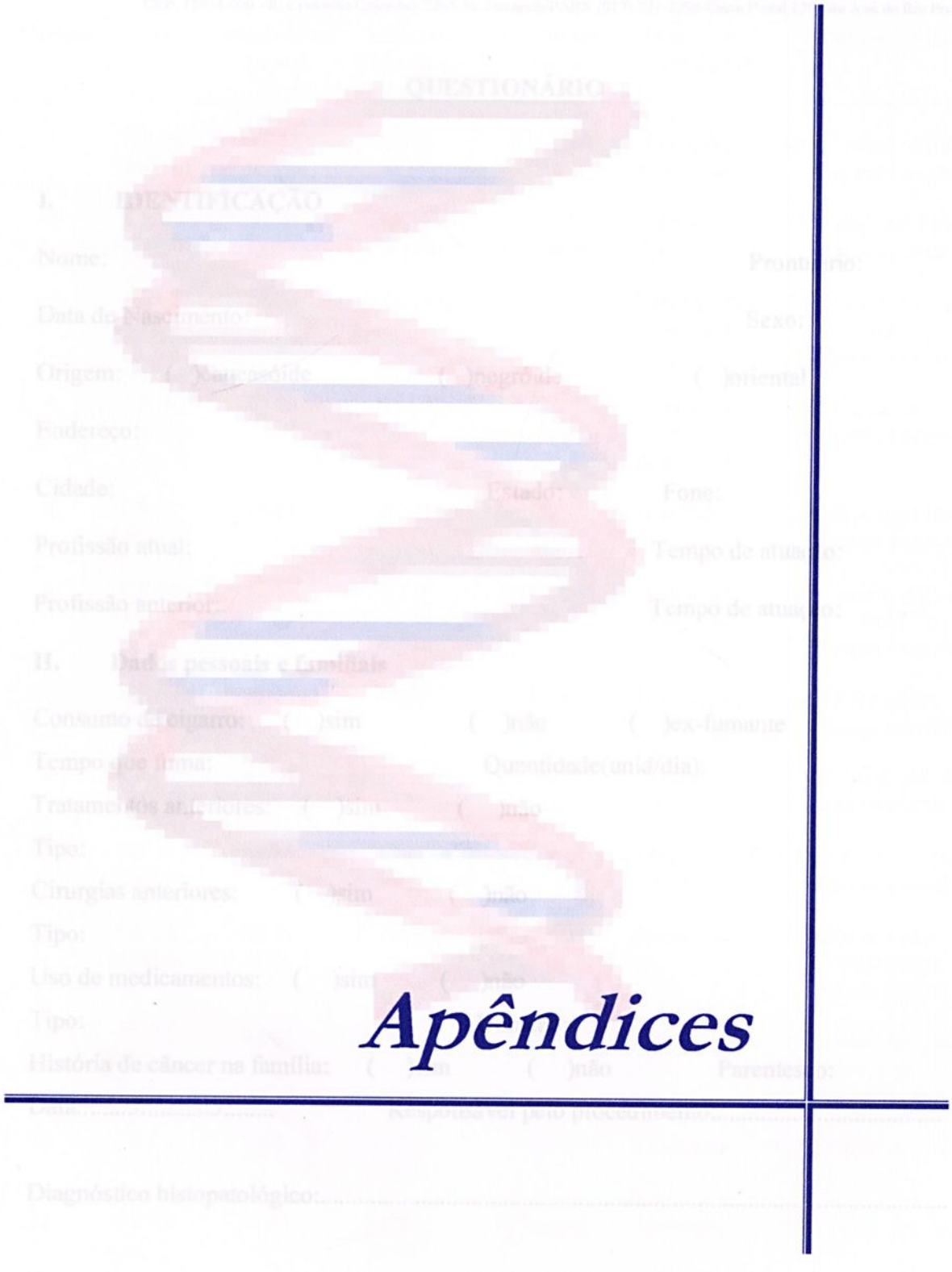
Nota: As refências citadas na introdução e discussão desta tese estão de acordo com as normas pulicadas pela ABNT de 2002, enquanto que as referências apresentadas nos artigos que compõem este trabalho estão de acordo com as normas propostas pelas respectivas revistas a que foram ou serão submetidos.

Associação Brasileira de Normas Técnicas. **NBR 6023**. Informação e Documentação: Referências – Elaboração. Rio de Janeiro, 2002.

Associação Brasileira de Normas Técnicas. **NBR 10520**. Informação e Documentação: Citações em documentos– Elaboração. Rio de Janeiro, 2002.

Associação Brasileira de Normas Técnicas. **NBR 14724**. Informação e Documentação: Trabalhos Acadêmicos– Elaboração. Rio de Janeiro, 2002.

Apêndices



Apêndices

UNESP UNIVERSIDADE ESTADUAL PAULISTA
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QUESTIONÁRIO

I. IDENTIFICAÇÃO

Nome: Prontuário:

Data de Nascimento: Sexo:

Origem: ( )caucasóide ( )negróide ( )oriental

Endereço:

Cidade: Estado: Fone:

Profissão atual: Tempo de atuação:

Profissão anterior: Tempo de atuação:

II. Dados pessoais e familiais

Consumo de cigarro: ( )sim ( )não ( )ex-fumante

Tempo que fuma: Quantidade(unid/dia):

Tratamentos anteriores: ( )sim ( )não

Tipo:

Cirurgias anteriores: ( )sim ( )não

Tipo:

Uso de medicamentos: ( )sim ( )não

Tipo: Duração:

História de câncer na família: ( )sim ( )não Parentesco:

Data...../...../..... Responsável pelo procedimento:.....

Diagnóstico histopatológico:.....

Tabela 1. Caracterização das amostras provenientes dos tumores e mucosa normal de esôfago quanto à idade, sexo, etilismo, tabagismo, ocupação profissional e diagnóstico histopatológico.

Ordem	Caso	Idade/Sexo (anos)	Etilismo/ Tabagismo (anos)	Morbidade (tempo)	Ocupação profissional	Histologia/Grau
1	CE 01 MNE 01	68/M	S(25)/ S(25)	S (1 ano)	Lavrador/ Aposentado	Adeno / mod. dif. / inv. Mucosa normal
2	CE 02 MNE 02	63/M	Ex (20)/ Ex (20)	S (2 meses)	Pintor/ Aposentado	CCE / mod. dif. / inv. / II Mucosa normal
3	CE 03 MNE 03	58/M	S / S (35)	N	Operador de máquinas	CCE / mod. dif. / ulc./ inv. Mucosa normal
4	CE 04 MNE 04	65/M	N / EX	N	Serviço Gerais/ Aposentado	CCE / mod. dif. / inv. Mucosa normal
5	CE 07 MNE 07	75/M	N / N	N	Lavrador	Adeno / mod. dif. Mucosa normal
6	CE 08 MNE 08	52/M	S / S	S (3 anos)	Lavrador	CCE / pouco dif. / ulc/ inv. Mucosa normal
7	CE 09 MNE 09	61/M	N / S	S (3 anos)	Funcionário público	CCE / in situ Mucosa normal
8	CE 10 MNE 10	61/M	Ex (15) /Ex (53)	S (5 meses)	Guarda	CCE / mod. dif. /IV Mucosa normal
9	CE 11 MNE 11	59/M	S(20) / S(40)	S (2 anos)	Pedreiro	CCE / mod. dif. / inv. Mucosa normal
10	CE 12 MNE 12	64/M	N / S(40)	S (15 dias)	Ambulante	Adeno / ulc. pT3/pN1/pM1 Mucosa normal
11	CE 13 MNE 13	46/M	S / S(32)	N	Marceneiro	CCE / mod. dif. / ulc. / inv. / III Mucosa normal
12	CE 14	74/F	Ex (65) / S (66)	N	Lavadeira	CCE / mod. dif. / inv.
13	CE 15	51/M	S (26) / S (26)	S (2 anos)	Motorista	CCE / mod. dif. /inv.

14	CE 16	62/M	S / S (40)	N	Pedreiro	CCE / ind. / inv.
15	CE 18	58/M	S (20) / S (15)	S (1 ano)	Lavrador	CCE / mod. dif. / ulc. / inv.
16	CE 19	72/M	S / S(50)	S (20 dias)	Marceneiro	CCE / mod. dif. /inv.
17	CE 20	80/F	N / N	S (1 mês)	Do lar	CCE / ulc. / inv.
18	CE 21	64/M	*	N	Servente	CCE / mod. dif. / ulc / inv.
19	CE 22	73/F	S / S (35)	N	Operador de máquinas	Adeno. / pouco dif. / ulc. / inv. pT3/pN2/pMx
20	CE 23	49/M	S / S(30)	N	Pedreiro	CCE / mod. dif. / inv.
21	CE 26	55/M	Ex (10) / S (40)	S (5 anos)	Pedreiro	CCE / mod. dif. / ulc. / inv.
22	CE 27	65/M	*	*	Jornalista	CCE / mod. dif./ ulc
23	CE 28	54/M	S(6)/S(20)	N	Vigia	CCE / mod. dif./ ulc./ IV
24	CE 29	60/M	S(40) / S(40)	S (1 mês)	Lavrador	CCE / pouco dif. / inv.
25	CE30	96/M	Ex/Ex	N	Aposentado	CCE / mod. dif. / inv.
26	CE 31	63/M	S(31)/S(30)	N	Lavrador	CCE / mod. dif. / ulc./ inv.
27	CE 32	73/F	N / S(50)	S (2 anos)	Pensionista	CCE / mod. dif. / Inv.
28	CE 34	69/M	S(45) / S (45)	S (1 ano)	Aposentado	CCE / mod. dif. / inv.
29	CE 35	54/M	*	S (5 anos)	Lavrador	CCE / mod. dif. / ulc./ inv.
30	CE 36	65/M	S(30) / S(35)	S (10 dias)	Aposentado	CCE / bem dif. / ulc.
31	CE 37	67/M	S/S	N	Funcionário público	Carcinoma de pequenas células
32	CE 38	83/F	S/S	N	Aposentada	CCE / mod.dif / inv.
33	CE 39	64/M	S (40) /	S (10 dias)	Cobrador	CCE / mod. dif. / ulc. /

			S(40)		onibus	inv.
34	CE 40	62/F	N/S (7)	S (2 anos)	Do lar	*
35	CE 41	51/M	S (1) / S (30)	S (10 dias)	Lavrador	CCE / mod. dif. / inv.
36	CE 42	58/M	S/S(30)	S	Garçom Aposentado	CCE / pouco dif. / Inv.
37	CE 43	61/M	N/S	N	Gari	CCE / mod. dif. /Inv. pT2/pN2/pM0
38	CE 44 MNE 44	53/M	N/S(48)	N	Montador	CCE / mod.dif. / ulc. /inv. Mucosa normal
39	CE 45	64/M	S(48)/S(48)	N	Inativo	CCE / mod. dif. / inv.
40	CE 46	66/M	S/S	N	Lavrador	CCE / mod. dif. / inv.

M = masculino; F = feminino; S=sim; N=não; ex= ex-fumante/etilista; CCE = carcinoma de células escamosas;
* = dado não disponível

Tabela 2. Caracterização das amostras provenientes dos tumores e mucosa normal de estômago quanto a idade, sexo, etilismo, tabagismo ocupação profissional e diagnóstico histopatológico.

Ordem	Caso	Idade/ Sexo	Etilismo/ Tabagismo (anos)	Ocupação profissional	Morbidade (anos)	HP	Histologia/Grau	Estadiamento
1	CG 04 MNG 04	63/M	Ex / S(20)	Aposentado	N	-	Carcinoma epidermóide II Mucosa normal	*
2	CG 07 MNG 07	58/M	N / N	Aposentado	S (2 meses)	-	Adenocarcinoma Difuso Mucosa normal	pT3/pN1/pMx
3	CG 08 MNG 08	82/M	N / Ex(45)	Lavrador/ Aposentado	N	-	Adenocarcinoma Intestinal Mucosa normal	PT2/pN1
4	CG 09 MNG 09	42/M	Ex(20) / Ex(25)	Lavrador/ Aposentado	N	-	Adenocarcinoma Difuso Mucosa nomal	pT3/N0/Mx
5	CG 10 MNG 10	53/F	N / N	Faxineira	N	-	Carcinoma/invasivo e ulcerado/ Intestinal Mucosa normal	pT3/pN1/pMx
6	CG 12	63/M	S(38) / S(65)	Lavrador	N	-	Adenocarcinoma Intestinal / IV	pT4/N1/Mx
7	CG 13	28/F	N / N	Do lar	S	+	Adenocarcinoma Difuso / IV	pT4/N2/
8	CG 14	83/M	S(65) / S(8)	Lavrador	N	+	Adenocarcinoma Difuso / III	pT3/N1
9	CG 15	44/M	S(29) /S(24)	Motorista	N	+	Adenocarcinoma Difuso / I	pT1/N0/M0
10	CG 16	93/M	S(10) / S(30)	Lavrador	N	+	Adeno/inv. Difuso	*
11	CG 17	58/M	N / S(32)	Auxiliar de limpeza	N	+	Adenocarcinoma Intestinal /IV	pT3/N1/M1

12	CG 18	62/M	S(20) / S(5)	Mecânico	S (4 anos)	-	Adenocarcinoma Intestinal	*
13	CG 19	62/M	S(30) / S(40)	Lavrador	S (1 mês)	-	Adenocarcinoma Intestinal / III	pT3/N2/M0
14	CG 21	72/M	S(45) / S(45)	Lavrador	S (3 meses)	+	Adenocarcinoma Intestinal /IV	pTx/Nx/M1
15	CG 23 MNG 23	74/M	*	Vigia/ Aposentado	S (1 mês)	-	Adenocarcinoma Intestinal Mucosa Normal	*
16	CG 24 MNG 24	38/M	N / S	Mecânico	N	-	Adenocarcinoma Intestinal Mucosa normal	pT4/pN2/pM1
17	CG 27 MNG 27	66/M	N / N	Lavrador	S (3 anos)	+	Adenocarcinoma Intestinal Mucosa normal	pT2/pN2/pMx
18	CG 28 MNG 28	43/F	N / N	Doméstica	N	-	Adenocarcinoma Difuso Mucosa Normal	pT2/N1/Mx
19	CG 30 MNG 30	54/M	S(15)/S(15)	Pedreiro	N	+	Adenocarcinoma/ difuso Mucosa Normal	pT4/pN2/pM4
20	CG 31 MNG 31	80/F	N/N	Lavradora/ Aposentada	N	*	Adenocarcinoma/ Difuso II Mucosa normal	pT3/pNx/pMx
21	CG 32 MNG 32	65/F	N/N	Doméstica	N	+	Adenocarcinoma/ difuso Mucosa normal	pT1/Nx/Mx
22	CG 35	66/M	S / S	Aposentado	S (1 mês)	-	Adenocarcinoma Intestinal /IV	pTx/Nx/M1
23	CG 36	51/M	S / S	Lavrador	N	+	Adenocarcinoma Difuso /IV	PT3/N0

24	CG 37	71/M	S / S	Aposentado	N	+	Adenocarcinoma Difuso /II	pT3/N0/M0
25	CG 38	38/F	N / N	Doméstica	S (1 ano)	+	Adenocarcinoma Difuso /IV	pT3/N1/M1
26	CG 48	52/M	S(9)/S(9)	Serralheiro	N	*	Adenocarcinoma Difuso /III	pT2/pN2/pMx
27	CG 49	89/M	S / S	Aposentado	S (1 ano)	-	Adenocarcinoma mod. dif Intestinal / III	pT2/N1/Mx
28	CG 50	67/F	N/N	Doméstica	N	-	Adenocarcinoma Intestinal	*
29	CG 51	57/M	S(20)/S(46)	Motorista	N	-	Adenocarcinoma Difuso	*
30	CG 52	78/M	N/Ex(70)	Aposentado	N	-	Adenocarcinoma Difuso	pT3/pN1/pMx
31	CG 53	64/M	S/S	Lavrador	N	-	Adenocarcinoma Difuso /III	pT3/N0/Mx
32	CG 54	65/M	N/Ex (50)	Aposentado	N	-	Adenocarcinoma Difuso	PT3/pN0/pMx
33	CG 55	73/F	N/N	Doméstica	S (1 ano)	+	Adenocarcinoma	*
34	CG 56 MNG 56	79/M	Ex / S	Lavrador	S (10 dias)	-	Adenocarcinoma Difuso Mucosa normal	pT3/pN1/pMx
35	CG 57 MNG 57	62/M	*	*	N	-	Adenocarcinoma Difuso Mucosa normal	pT3/pN2/pMx
36	CG 58	64/F	N/N	Costureira	N	-	Adenocarcinoma Difuso	PT3/pN2/pMx
37	CG 59	57/M	*	Auxiliar Serviços Gerais	*	+	Adenocarcinoma Difuso	*

38	CG 60	84/F	N/N	Aposentada	N	-	Adenocarcinoma Difuso	*
39	CG 61	74/M	N/N	Aposentado	N	-	Adenocarcinoma Difuso	*
40	CG 62	82/M	*	Aposentado	N	-	Adenocarcinoma Difuso	*

M = masculino; F=feminino; S=sim, N=não; ex= ex-fumante/etilista; * = dado não disponível.


cm 1 2 3 4 5 6 7 8 unesp 12 13 14 15 16 17 18 19