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Research Article

# Gene amplification in carcinogenesis

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## Abstract

Gene amplification increases the number of genes in a genome and can give rise to karyotype abnormalities called double minutes (DM) and homogeneously staining regions (HSR), both of which have been widely observed in human tumors but are also known to play a major role during embryonic development due to the fact that they are responsible for the programmed increase of gene expression. The etiology of gene amplification during carcinogenesis is not yet completely understood but can be considered a result of genetic instability. Gene amplification leads to an increase in protein expression and provides a selective advantage during cell growth. Oncogenes such as *CCND1*, *c-MET*, *c-MYC*, *ERBB2*, *EGFR* and *MDM2* are amplified in human tumors and can be associated with 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. This review discusses the major role of gene amplification in the progression of carcinomas, formation of genetic markers and as possible therapeutic targets for the development of drugs for the treatment of some types of tumors.

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

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## Gene Amplification

### Structural characteristics of amplified genes

Gene amplification can be defined as an expansion in the number of copies of a gene in the cell genome which occurs by replication of the genomic DNA to produce karyotype abnormalities called double minutes (DM) and homogeneously staining regions (HSR) (Bast *et al.*, 2000; Simon *et al.*, 2002). Such gene amplification should be considered separately from chromosome aneuploidy, which is outside the scope of this review.

Double minutes are extrachromosomal circles of DNA containing 1 to 2 million base pairs which replicate autonomously about once every cell cycle and, because they have no centromeres, segregate at random to the daughter cells. In contrast, homogeneously staining regions are intrachromosomal segments forming large genomic regions (Hellman *et al.*, 2002) which display no chromosome banding pattern when submitted to G-banding but can be detected by fluorescence *in situ* hybridization (FISH). Both these structures have amplified genomic DNA, containing hundreds of copies of one or more genes, frequently oncogenes (Bast *et al.*, 2000).

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### Gene amplification during development

Gene amplification plays a major role during development when it is responsible for the programmed increase of gene expression. For example, because amphibian oocytes require high levels of proteins genes responsible for ribosomal RNA (rRNA) are amplified to produce between two thousand and one million copies of rRNA per oocyte. Other examples are the amplification of genes responsible for chorionic proteins in *Drosophila* oocytes (Cooper, 2000) and the amplification of the *LAC* allele region in *Escherichia coli* growing under conditions of environmental stress such as lactose deficiency, such amplification resulting in higher production of the enzyme lactase enabling the bacteria to grow even in a restrictive environment (Hastings and Rosenberg, 2002).

### Gene amplification in carcinogenesis

Gene amplification involving double minutes and homogeneously staining regions has been widely observed in human tumors in which these processes act 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). Exact data on the frequency of double minutes and homogeneously staining regions in tumor cells *in vivo* are difficult to obtain since these abnormalities are easily missed in routine cytogenetic analysis. Also, the percentage of cells expressing double minutes or homogeneously staining regions varies widely. Both events are seen more frequently in established cell lines than in primary tumors (Schwab *et al*., 1999) and homogeneously staining regions more frequently in advanced stages of tumors (Schwab *et al*., 1999).

The etiology of gene amplification in cancer is not yet completely understood, but it can be considered a result of genetic instability. Cytogenetic studies of rat drug resistance genes indicate that chromosome breaks followed by fusion of distant segments and the consequent formation of anaphase bridges frequently occur in tumor cells (Hellman *et al.*, 2002), this process being particularly common at certain fragile sites which are unstable chromosomic regions prone to breaks or gaps during cell division. Hypoxia is a powerful fragile site inducer and is thought to facilitate the fusion of double minutes and their reintegration into the chromosomal fragile sites to produce homogeneously staining regions (Ishizuka *et al.*, 2002).

Gene amplification leads to an increase in gene expression and this can confer a selective advantage during the early stages of the neoplastic process. However, amplification may also occur as a late tumorigenesis event (Tsujimoto *et al.*, 1997; Bast *et al.*, 2000; Sarasin, 2003). Although gene amplification appears to be the main mechanism leading to protein overexpression in carcinogenesis the gain of gene copies by polysomy may also result in high protein levels, as has been reported for breast (Lal *et al*., 2003), esophageal and gastric carcinomas with positive immuno-staining but no amplification (Bizari *et al.*, in press).

## Main Amplified Genes in Human Neoplasias

Several studies have shown that some oncogenes (*e.g.* *CCND1*, *c-MET*, c-*MYC*, *ERBB2*, *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).

The *MYCN* oncogene localized at 2p23-24 is a member of the MYC-box group of genes (Schwab *et al.*, 1988) and encodes a 65 kDa transcriptional factor (Ramsay *et al.*, 1986; Slamon *et al.*, 1986; Schwab *et al.*, 2004). The basic mechanism for MYCN protein activity involves formation of a mandatory heterodimer with a nuclear phosphoprotein called MAX (Facchini *et al*., 1998). This heterodimer binds to specific DNA E-box elements to initiate target gene transcription. Due to its association with aggressively growing tumor phenotypes *MYCN* was the first clinically significance oncogene amplified (Schwab *et al.*, 1995). Amplification of the *MYCN* oncogene occurs as double minutes or homogeneously staining regions and has been found only in more aggressive variants of neuroblastoma where it indicates a bad clinical prognosis (Rubie *et al.*, 1997; Solovei *et al.*, 2000; Perel *et al.*, 2004; Schwab *et al.*, 2004). Conversely, the expression of *MYCN* without amplification is a normal feature of cells of various tissues including retinal (Squire *et al.*, 1986) and kidney (Zimmerman *et al.*, 1986) tissue. In neuroblastoma no correlation between *MYCN* overexpression and amplification has been observed (Vasudevan *et al.*, 2005).

The *MYCN* amplicon can be up to 1 Mb in size and so could contain additional genes that affect tumor phenotype. The *DDX1* gene is frequently co-amplified with *MYCN* in neuroblastoma (Scott *et al.*, 2003) and in 50-70% of different primary tumors and in about 70% of cell lines (George *et al.*, 2000; Preter *et al.*, 2002). The *DDX1* gene maps 340 kb telomeric to the *MYCN* gene (George *et al.*, 2000) and encodes a 2.7-kb transcript with a predicted protein product of 740 amino acids. A poorer prognosis has been observed with *DDX1* co-amplification (Squire *et al.*, 1995) but other workers have reported no significant difference in clinical presentation or outcome (Manohar *et al.* 1995) or even a better prognosis and improved patient survival (Weber *et al.*, 2004).

Other genes co-amplified in tumors include *MDM2* and *CDK4*, mapped at 12q14-15 (Wunder *et al.*, 2000; Lopez-Guerrero *et al.*, 2004; Pedeutour *et al.*, 2004) and *CCND*, *INT-2*, *EMS1* and *HST-1*, mapped at 11q13. The 11q13 amplicon can vary in size from less than 1 to 4.5Mb and recent data have identified four core regions within 11q13 that can be amplified independently or together in different combinations (Tanner *et al*., 1996; Ormandy *et al*., 2003). 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 belonging to the cyclin D family which includes cyclins D2 and D3 (Ewen and Lamb, 2004). The D1 cyclin regulates the transition of cells from the G1 phase to the S phase by phosphorylation of the pRb protein during the G1 phase and the release of 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 cell cycle transition (Ewen and Lamb, 2004). Cyclin D1 acts by form-

ing the D1 cyclin/cyclin-dependent protein kinase (CDK) complex D1-CDK which activates specific CDKs (CDK4 and CDK6) which allow the cell cycle to progress (Rabbani *et al.*, 2000; Saikawa *et al.*, 2001) (Figure 1). Amplification of the gene encoding the D1 cyclin is seen in a variety of solid tumors, including breast adenocarcinoma, squamous cell carcinoma of head and neck, esophageal and bladder cancer (Ewen and Lamb, 2004). In bladder tumors, overexpression of the D1 cyclin protein is correlated with high-degree tumors and a short recurrence time but no association between tumor invasion or decreased survival and Cyclin D1 overexpression has been detected (Adshead *et al.*, 1998).

Amplification of the *CCND1* oncogene also occurs in the early stages of these tumors and persists during the advanced stages of the disease, although the exact mechanism leading to progression remains unclear (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 esophageal tumors to chemotherapeutic agents (Nagasawa *et al.*, 2001). In esophageal carcinomas, it has been shown that gene amplification of homogeneously staining regions, as opposed to polysomy, was the main mechanism responsible for overexpression of the Cyclin D1 protein (Bizari e*t al*., in press).

In breast cancer, both gene amplification and overexpression of the protein Cyclin D1 are related to a shorter survival time, a shorter period free of the disease and a higher tumor recurrence rate (Ormandy *et al.*, 2003).

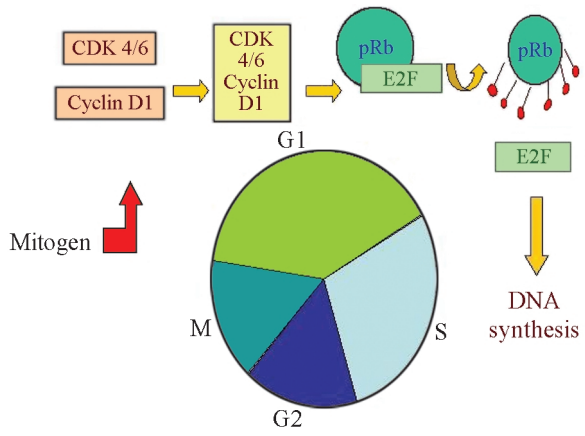


**Figure 1** - Simplified G1/S regulation. When a dividing cell is about to go through another round of division, the Rb protein (or its related family members) is phosphorylated by cyclin/CDK complex which releases the E2F transcription factor and leads to changes in gene expression that are essential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

Another widely studied amplicon is located in region 7q31-q32 and includes the c-*MET* oncogene which 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 (especially for diffuse tumors) and is never found in the early stages of carcinogenesis, because of which the *c-MET* oncogene is a marker of poor prognosis and indicates progression and increased 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 region 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 including those encoding ornithine decarboxylase, phosphatase cdc25A and the transcription factor E2F (Zajac-Kaye, 2001; Mai *et al.*, 2003) and repressing others (such as the p27 releasing protein from the cdk2 complex) by means of the activity of the cyclin E-cdk2 complex. Once released, the p27 protein is sequestered by the cdk4-cyclin D complex to form a new complex (cdk4-cyclinD-p27) able to phosphorylate pRb and release the E2F transcription factor enabling the cell to perform the G1-S cell cycle transition (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), although expression of this protein usually 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) *c-MYC* amplification 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) but in bladder carcinomas such a correlation is not yet clear, and 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).

The *ERBB2* gene, also known as *HER-2/neu*, is mapped to region 17q11-q12 (Yamamoto *et al.*, 1986) and is amplified in tumors of various tissues such as breast, ovary, bladder, stomach and lung (Takehana *et al.*, 2002; Lear-

Kaul *et al.*, 2003) and is a potential marker of prognosis in some them. 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 cancer cell lines, the repetitive unit of the *ERBB2* amplicon was identified as a 120 kilobase strech of genomic DNA with structure of homogeneously staining regions (Dahlberg *et al*., 2004) (Figure 3). In breast cancer, the 17q11 amplicon harbors the *ERBB2* gene and other putative oncogenes (Barlund *et al*., 1997; Sinclair *et al*., 2003).

In breast and lung tumors, *ERBB2* 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), while overexpression in gastric tumors is related to the presence of metastases (Nessling *et al.*, 1998; Vidgren *et al.*, 1999; Varis *et al.*, 2002) and evolution to the gastric intestinal type (Becker *et al.*, 2000). Evaluation of *ERBB2* gene status has revealed that in both esophageal and gastric cancer high-polysomy, as opposed to gene amplification, was the prevalent pattern regarding a gain in *ERBB2* gene copies (Bizari *et al*., in press; Sunpaweravong *et al*., 2005).

Amplification of the *ERBB2* oncogene, 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 tumors as well as in breast tumors, amplification and overexpression of the c-erbB2/neu protein has been investigated with the purpose of possibly directing the treatment of positive patients towards the use of the monoclonal antibody Herceptin (trastuzumab), since the levels of gene amplification and protein expression are very similar to those found in patients with breast cancer (Ross and McKenna, 2001; Ross and Gray, 2003). Herceptin works as a negative regulator of the c-erbB2/neu protein, inhibiting the signaling pathway of the cell cycle and blocking its transition. *In vivo*, herceptin 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 *c-MYC* and *c-MET* oncogenes (Ma *et al.*, 2003). The c-myc protein 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 tumor regression (Hermeking *et al.*, 2003).

In conclusion, detailed analyses of genomic structures and sequences of amplified regions have revealed that amplicons exhibit complex patterns and can harbor multiple genes associated with tumorigenesis. The evaluation of

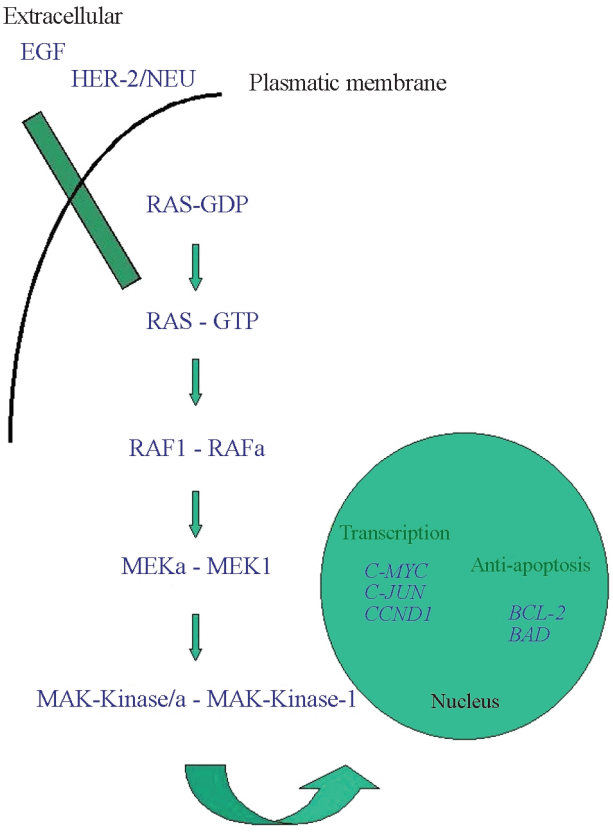


**Figure 2** - Activation of epidermal growth factor receptor (EGFR) results in the initiation of a diverse array of cellular pathways. Dimerization results in autophosphorylation, initiating a downstream cascade of events and culminating in cellular responses such as cell proliferation or apoptosis. These protein interactions allowing Ras activation which 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.

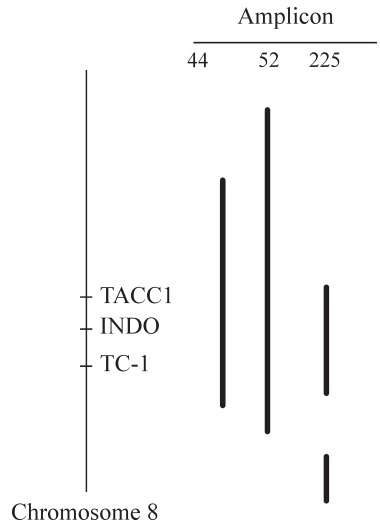


**Figure 3** - Ideogram of the 8p11-p12 amplicon in three HBC cell lines, SUM-44, SUM-52 and SUM-225 showing the conserved region (modified from Yang *et al.*, 2004).

co-amplified genes may provide important insights into the pathogenesis of cancer, and can lead to the identification of targets for novel therapeutics. Gene amplification as double minutes and homogeneously staining regions offers the opportunity for functional studies of candidate oncogenes and also to analyze the factors which drive the neoplastic process, the number of mutations required for malignancy and the selective advantage which tumor cells have acquired during tumorigenesis. For example, comparison of the 8p11-p12 amplicon in different breast cancer cell lines showed a conserved region that may contain more than one important gene for tumor development, including the *TACC1*, *INDO* and *TC-1* genes (Sunde *et al*., 2004) (Figure 3). The *TACC1* gene participates in anchorage-independent growth (Lapin *et al*., 2002) while *INDO* mediates immunosuppression (Li *et al*., 2004) and *TC-1* is related to apoptotic process during the carcinogenic process (Sunde *et al*., 2004).

The potential of double minute and homogeneously staining region analysis is far from exhausted. Comparison of amplicons from different samples, fine mapping of the amplified region and functional studies may reveal new pathways associated with tumor growth and the clinical course of the disease. The results can certainly provide new tools in the fight against cancer.

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