CHROMOSOME 6q DELETIONS IN GASTRIC CARCINOMA

A positional cloning approach to isolate cDNAs at 6q16.3-q23.3

DELECÇÕES DO BRAÇO LONGO DO CROMOSSOMA 6 EM CARCINOMAS GÁSTRICOS

Isolamento de cDNAs em 6q16.3-q23.3 por clonagem posicional

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NOTA EXPLICATIVA

A presente dissertação está escrita em inglês na sua quase totalidade, exceptuando o Sumário e Conclusões, pelo facto de o Doutor Klaas Kok ter sido o seu orientador.

TABLE OF CONTENTS

ABBREVIATIONS

AI – Allelic imbalance

ALL – Acute lymphoblastic leukemia

AML – Acute myeloid leukemia

BAC – Bacterial artificial chromosome

bp – Base pair

cDNA – Complementary DNA

CGH – Comparative genomic hybridization

CIN – Chromosomal instability pathway

cM – CentiMorgan

DD – Differential display

DNA – Deoxyribonucleic acid

ESD – Equalization of cDNAs; subtractive hybridization; differential display

EST – Expression sequence tag

IPGI – Integrated procedure for gene identification

Kb – Kilobase

LOH – Loss of heterozygosity

Mb – Megabase

MIN – Hypermutability pathway

MLL – Myeloid lymphoblastic leukemia

MMPs – Metalloproteinases

mRNA – Messenger RNA

MSI – Microsatellite instability

MSS – Microsatellite stable

PAC – P1 artificial chromosome

PCR – Polymerase chain reaction

PFGE – Pulsed-field gel electrophoresis

Ph – Philadelphia chromosome

RDA – Representational difference analysis

RER – Replication error

RFLP – Restriction fragment length polymorphism

RNA – Ribonucleic acid

SAGE – Serial analysis of gene expression

SRO – Smallest region of overlap

SSH – Suppression subtractive hybridization

STS – Sequence tagged site

TSG – Tumor suppressor gene

VNTR – Variable number of tandem repeats

YAC – Yeast artificial chromosome

INTRODUCTION

1. Genetics of cancer

Tumor formation results from the accumulation of genetic alterations that disrupt the control of cell proliferation. This genetic instability of tumors may reflect two different underlying mechanisms: the hypermutability pathway (MIN), where instability occurs at the nucleotide level, resulting in base substitutions, deletions or insertions of a few nucleotides; and the chromosomal instability pathway (CIN), where the instability occurs at the chromosome level resulting in losses and gains of large portions or whole chromosomes (Boland, 1997; Lengauer *et al*, 1997; Lengauer *et al*, 1998). The MIN pathway occurs in a small subset of tumors whereas CIN occurs in most other cancers (Lengauer *et al*, 1998). In general, there is little overlap between MIN and CIN pathways, although in late-stage cancers, the two mechanisms may converge (Boland, 1997).

In the hypermutability pathway the instability observed is a consequence of the inactivation of repair genes that become unable to correct replication errors (MSI phenotype), leading to an increase of the mutation rate and affecting not only non-coding DNA but also intragenic regions (Boland, 1997; Wang *et al*, 1997; Oliveira *et al*, 1998; Iacopetta *et al*, 1999).

In the chromosomal instability pathway, the gross chromosomal alterations result in aneuploidy of cancer cells and lead to activation of oncogenes and inactivation of tumor suppressor genes (Levine, 1993; Rabbitts, 1994; Boland, 1997; Dos Santos and van Kessel, 1999).

More recently, genes involved in tumorigenesis are classified in gatekeepers and caretakers (Kinzler and Vogelstein, 1997). Gatekeepers prevent tumorigenesis by inhibiting cell growth or promoting cell death and caretakers allow cancer development indirectly, as their dysfunction leads to genetic instability, favoring mutations in others genes, including gatekeepers.

1.1. Oncogenes and tumor suppressor genes

Oncogenes are altered forms of normal cellular genes, also called proto-oncogenes. Proto-oncogenes are growth-promoting genes which stimulate, when expressed, cell proliferation. On the other hand, there is a group of genes that regulate cell proliferation

in a negative way. These genes are named tumor suppressor genes (TSGs). The equilibrium between these two types of genes allows the homeostasis of cells.

A single mutational event in a proto-oncogene may be sufficient to activate it, allowing its oncogenic product to contribute to the abnormal growth of cells (Levine, 1993). An example of oncogene activation is a point mutation that results in a hyper-functioning protein, as it happens in the *RAS* family of oncogenes (Park, 1998). Activation of an oncogene can also be achieved by a chromosomal translocation or inversion that either juxtaposes the oncogene to an actively transcribing gene or produces a fusion gene (Rabbitts, 1994; Dos Santos and van Kessel, 1999). *C-MYC* translocation in Burkitt's lymphoma is an example of activation of an oncogene by juxtaposition with an immunoglobulin (or T-cell receptor) gene, that is naturally rearranged to generate active antigen receptor genes (Rabbitts, 1994). The consequence of this rearrangement is that *C-MYC* becomes actively transcribed. On the other hand, the Philadelphia (Ph) chromosome in chronic myelogenous leukemia results from the translocation between chromosomes 9 and 22, leading to the fusion of *BCR* and *ABL* genes and consequently to the expression of an oncogenic fusion protein (Rabbitts, 1994).

Chromosomal amplifications of regions containing one or more oncogenes lead to a dosage increase of these genes resulting in their overexpression, as it is observed with *N-MYC* oncogene in neuroblastomas (Dos Santos and van Kessel, 1999).

As tumor suppressor genes have a negative role in the regulation of cell proliferation, they will only contribute to the abnormal growth of cells when both alleles are inactivated. Thus, two independent inactivating mutational events are required to achieve this (Levine, 1993).

Three lines of evidence suggested the existence of tumor suppressor genes: studies with hybrid cells, of familial cancers and of loss of heterozygosity (LOH) in sporadic tumors (Marshall, 1991). The fusion of a cancer cell with a normal cell gives rise to a hybrid cell line no longer capable of forming tumors in animals. Also, the fusion of a microcell, containing a single normal chromosome, with a tumorigenic cell can lead to the suppression of tumorigenicity. These experiments showed that the function of a tumor suppressor gene can be restored by the introduction of a single wild-type allele, and thus show that in cancer cells both alleles are inactivated (Levine, 1993).

As proposed by Knudson (1971) in his two-hit model, on the study of retinoblastoma, the first hit consists of a somatic mutation (or a germline mutation in case of familial cancer) and the second hit is the loss of the remaining allele, reducing the mutant allele to homozygosity. Together with the loss of the second allele, flanking chromosomal regions may also be lost, suffering a parallel reduction to homozygosity, or loss of heterozygosity (LOH). Indeed, the consistent observation of LOH of a specific chromosomal region in cells from a particular tumor type suggests the presence of a tumor suppressor gene in that region, whose functional loss is involved in tumor development (Weinberg, 1991).

Retinoblastoma gene (*RB1*) and *TP53* are paradigmatic examples of tumor suppressor genes, that are involved in the regulation of the cell-cycle (Levine *et al*, 1993).

The loss of function of the second allele of a tumor suppressor gene can also be caused by methylation, leading to the silencing of that allele. Examples of tumor suppressor genes that can be inactivated by this mechanism are the *E-cadherin* (Graff *et al*, 1995) and $p16^{INK4}$ (Song *et al*, 2000) genes.

2. Gastric carcinoma

Despite the overall decreasing rates of incidence and mortality, gastric cancer remains the fourth most common cause of cancer-related death in the European community (Black *et al*, 1997) and the leading one in Portugal (Da Motta, 1994). The incidence rate (31.9 per 100,000) in Portugal is the highest, being approximately twice the average for the European Union (Black *et al*, 1997). In this view, it is of major importance the study of gastric cancer and the understanding of the mechanisms underlying the disease development.

Environmental factors are thought to have influence in gastric cancer development, such as diets with high salt content and poor in fruit and vegetables, and bacterial infection with *Helicobacter pylori* (Fuchs and Mayer, 1995; Black *et al*, 1997). The changing of diet habits and the improvement of food preservation are thought to explain the decline observed in the prevalence of this disease in most countries (Fuchs and Mayer, 1995; Black *et al*, 1997).

Besides environmental factors, host susceptibility and genetic factors play a role in gastric carcinoma development. It was shown that there is a lower incidence of

adenocarcinomas in individuals from the blood group O and B, compared to A individuals (Mourant *et al*, 1978). In an epidemiological study, where the relationship between *MUC1* gene polymorphism and gastric cancer was evaluated (Carvalho *et al*, 1997), it was shown that individuals with small genotypes for *MUC1* were at increased risk for gastric carcinoma development. Recently, it was shown that genotypes of the *IL-1* gene cluster that enhance IL-1β production (a pro-inflammatory cytokine) increase the likelihood of a chronic hypochlorhydric response to *Helicobacter pylori* infection and the risk of gastric cancer (El-Omar *et al*, 2000).

There are two major histologic types of gastric carcinoma: the intestinal-type (Laurén, 1965) or glandular carcinoma (Carneiro, 1997) and the diffuse-type (Laurén, 1965) or isolated-cell type carcinoma (Carneiro, 1997). The first type is characterized by cohesive cells forming a gland-like structure, has an expansive growth and gives rise to blood born metastases. In contrast, the diffuse-type is characterized by isolated cells dispersed in the stroma, has an infiltrating growth and tends to invade the surrounding tissues and to disseminate into the peritoneum and to the regional lymph nodes (through the lymphatic vessels). The intestinal carcinomas usually occur in the distal stomach, in elder patients and more often in geographic regions with high risk of gastric carcinoma (where diet and infection play a role). The diffuse carcinomas occur throughout the stomach, in younger patients and its incidence is similar in most populations throughout the world. These two entities seem to have different genetic pathways underlying the different phenotypic outcome (Correa, 1992; Fuchs and Mayer, 1995; Carneiro, 1997).

2.1. Genetic events in gastric carcinoma

Several chromosomes and genes have been implicated in the development and progression of hereditary and sporadic gastric cancer, sometimes with conflicting results between different groups. However, some genes were already shown to have an unquestionable role in gastric cancer development like *E-cadherin* in the diffuse histotype of gastric carcinoma, both in sporadic and in hereditary forms (Becker *et al*, 1994; Guilford *et al*, 1998; Machado *et al*, 1999). Below, a brief review is made of the genetic alterations described in the past years.

2.1.1. Classic cytogenetic analysis

In vitro culture of gastric carcinoma cells is difficult, specially in the isolated cell type, where fibroblasts tend to overgrow tumor cells (Gärtner *et al*, 1996). As in many solid tumors, gastric cancer karyotypes are very complex (Rodriguez *et al*, 1994). Usually, the tumors that are analyzed are in a late stage of progression, showing numerous and complex chromosomal abnormalities.

Nevertheless, there are a few cytogenetic studies in gastric carcinomas that pinpointed several chromosomal abnormalities, both numerical and structural, which became good candidate sites for molecular characterization.

Polysomies of chromosomes 2 and 20 (Seruca *et al*, 1993), as well as 16 (Trigo *et al*, 1994), were found. Also, trisomies of chromosomes 8, 19 (Xiao *et al*, 1992; Trigo *et al*, 1994; Panani *et al*, 1995) and 12 (Ochi *et al*, 1986) were described. Chromosomes more often involved in structural rearrangements are chromosomes 1 (Ochi *et al*, 1986; Seruca *et al*, 1993; Panani *et al*, 1995; Chun *et al*, 2000), 3 (Ochi *et al*, 1986; Xiao *et al*, 1992; Seruca *et al*, 1993; Panani *et al*, 1995), 6 (Ochi *et al*, 1986; Seruca *et al*, 1993; Barletta *et al*, 1993; Panani *et al*, 1995) and 7 (Ochi *et al*, 1986; Seruca *et al*; 1993; Xiao *et al*, 1992; Chun *et al*, 2000). Also, chromosomes 5 (Barletta *et al*, 1993; Panani *et al*, 1995; Chun *et al*, 2000), 13 (Seruca *et al*; 1993; Panani *et al*, 1995), 19 (Ochi *et al*, 1986; Barletta *et al*, 1993) 12 and 20 (Ochi *et al*, 1986; Panani *et al*, 1995) were described as being involved in structural aberrations in gastric carcinoma. Some breakpoints are recurrently involved, like 1p22 (Ochi *et al*, 1986; Seruca *et al*; 1993), 3p21 (Ochi *et al*, 1986; Seruca *et al*; 1993), 6q21 (Ochi *et al*, 1986; Seruca *et al*; 1993; Panani *et al*, 1995) and 13q14 (Seruca *et al*; 1993), suggesting the presence of relevant genes in these regions.

Although a preferential involvement of certain chromosomes has been described, as shown above, putatively representing primary cytogenetic abnormalities, data are very limited to draw conclusions about specific cytogenetic abnormalities in gastric cancer.

2.1.2. Molecular cytogenetic analysis

A method that overcomes the difficulties in obtaining good quality metaphases in solid tumors is comparative genomic hybridization – CGH (Kallioniemi *et al*, 1992). This method is based on the *in situ* hybridization of differentially labeled total genomic

tumor and reference DNAs to normal human metaphases. A single hybridization allows the analysis of the entire genome for increases and decreases in DNA copy number in the tumor and maps these changes on normal chromosomes.

Results obtained by CGH in gastric carcinomas showed that the chromosomal regions where a gain of material is commonly observed are 7p, 7q, 8q, 17q and 20q (Kokkola *et al*, 1997; El-Rifai *et al*, 1998; Nessling *et al*, 1998; Sakakura *et al*, 1999; Okada *et al*, 2000; Koo *et al*, 2000), with regions 7q, 17q and 20q showing more frequently high levels of amplification (Kokkola *et al*, 1997; Nessling *et al*, 1998; Sakakura *et al*, 1999).

Losses detected by this technique usually involve chromosome arms 1p, 3p, 5q, 6q, 17p and 18q (Koizumi *et al*, 1997; Kokkola *et al*, 1997; El-Rifai *et al*, 1998; Nessling *et al*, 1998; Sakakura *et al*, 1999; Koo *et al*, 2000; Okada *et al*, 2000).

2.1.3. Loss of heterozygosity studies

Loss of heterozygosity (LOH) studies in different series of gastric carcinomas showed that many chromosomal regions are heterozygously deleted in this malignancy. Many of the chromosome arms involved are coincident with the data obtained by conventional cytogenetics and CGH.

Chromosome arms 1p, 3p, 5q, 6q, 11p, 11q, 13q, 17p and 18q are often found to be frequently deleted (Uchino *et al*, 1992; Seruca *et al*, 1995a; Schneider *et al*, 1995; Baffa *et al*, 1996; Ezaki *et al*, 1996; Tamura *et al*, 1996a; Gleeson *et al*, 1997; Choi *et al*, 1998; Yustein *et al*, 1999). Deletions in chromosome arms 4q, 7q, 16q and 21q were also reported (Uchino *et al*, 1992; Tamura *et al*, 1996b; Gleeson *et al*, 1997; Mori *et al*, 1999). Frequencies of LOH vary from study to study, depending on which markers and number of cases were analyzed, hampering an accurate comparison of the results.

Some of the deletions frequently involved in gastric cancer development are common to both types of gastric carcinoma (intestinal and diffuse), like 1p, 3p, 6q, 13q, 18q (Seruca *et al*, 1995a; Schneider *et al*, 1995; Cho *et al*, 1996; Gleeson *et al*, 1997; Choi *et al*, 1998; Fang *et al*, 1998; Yustein *et al*, 1999). There is some discrepancy regarding deletions in the short arm of chromosome 17. Some authors argue that these are common to both histotypes (Schneider *et al*, 1995; Gleeson *et al*, 1997; Choi *et al*, 1998) whereas others argue that 17p deletions are rarely found in diffuse cancers (Seruca *et al*, 1995a; Kobayashi *et al*, 1996). Also 5q deletions give controversial

results. While some defend that there is no histological association (Cho *et al*, 1996; Gleeson *et al*, 1997; Yustein *et al*, 1999), others contend that there is an association with intestinal carcinomas (Seruca *et al*, 1995a; Wu *et al*, 1997a; Wu *et al*, 1998).

Some of the deletions described above are detected in early stages of development, like 3p, 6q, 13q, 17p, but some seem related with progression (only detected in advanced carcinomas), like 5q and 18q (Seruca *et al*, 1995a; Nishizuka *et al*, 1998).

2.1.4. Comparison of classical cytogenetics, CGH and LOH data

Comparison of the literature data on structural aberrations detected by cytogenetics with CGH and LOH, shows that losses at 1p, 3p, 5q, 6q, 13q, 17p and 18q, and gains of chromosome 20, are the most frequent imbalances in gastric cancer.

2.1.5. Genes in gastric cancer

Several tumor suppressor genes, oncogenes and metastasis-related genes have been implicated in gastric carcinogenesis. The different pattern of gene alterations detected in the two main histologic types of stomach cancer (intestinal and diffuse type), as will be discussed below, supports the existence of two different genetic pathways underlying these two main entities (Tahara *et al*, 1996a; Carneiro, 1997).

2.1.5.1. Tumor suppressor genes

The classical form of inactivation of tumor suppressor genes (TSGs), implies mutation of one allele and loss of the remaining wild-type allele.

TP53 (located at 17p13), one of the genes most frequently implicated in cancer in general, is also altered (Tahara *et al*, 1996a) in both intestinal and diffuse types of gastric carcinoma. As shown by Strickler *et al* (1994), *TP53* mutations occur in early stages of development, before clonal expansion.

The *APC* gene, located at 5q21, is frequently mutated in colon cancer (Fearon and Vogelstein, 1990). In gastric cancer, the *APC locus* is frequently deleted in carcinomas of the intestinal histologic type but not in the diffuse histotype (Seruca *et al*, 1995a). Sanz-Ortega *et al* (1996) showed that deletions of this *locus* occurred both in early and in advanced cases and were already detected in dysplastic lesions and metaplasias,

suggesting that, as with *TP53*, alterations of *APC* might play a role in early stages of gastric carcinogenesis. However, inactivating mutations of the remaining allele of the *APC* gene were infrequently found in gastric cancer (Ogasawara *et al*, 1994; Powell *et al*, 1996). Thus, the role of *APC* inactivation in gastric carcinoma is still not clear.

Other TSGs inactivated in gastric cancer are the cyclin-dependent kinase (CDK) inhibitors, *p21* $^{waf1/cip1}$ and *p16* ^{INK4} (Tahara *et al*, 1996a). Here, the loss of function of one of the alleles appears to be caused by hypermethylation of the promoter region. Loss of *p16* ^{INK4} expression by methylation of CpG sites in the promoter region has been demonstrated in gastric cancer (Song *et al*, 2000; Shim *et al*, 2000).

Other examples of TSGs putatively implicated in gastric cancer development are genes located in chromosomal regions demonstrated to be lost in gastric cancer, like 3p and 18q.

FHIT (Fragile histidine triad), located at chromosome region 3p14, has recently been proposed as a tumor suppressor gene (Ohta *et al*, 1996), but its role in gastric carcinogenesis remains controversial. Baffa *et al* (1998) detected deletions or rearrangements of this gene in gastric tumors and cell lines with loss of expression in 67% of cases, suggesting a role of this gene in gastric tumorigenesis. On the other hand, Noguchi *et al* (1999) found LOH in only 16% of informative cases in a series of 133 gastric cancers and did not find a correlation with any clinic-pathologic parameter.

Recently, the *SMAD4* gene (deleted in pancreatic cancer), located at 18q, was implicated as a TSG in gastric carcinogenesis (Takaku *et al*, 1999; Xu *et al*, 2000). It was shown that mice with *SMAD4* haploinsufficiency (+/- for *SMAD4*) developed polyps in the antrum that progressed to dysplasia and to *in situ* carcinoma, and, eventually, to invasive carcinoma. Loss of the second allele occurred in later stages of progression.

E-cadherin, previously pinpointed as a TSG involved in breast cancer development (Berx *et al*, 1995; Graff *et al*, 1995), was shown to be associated with the diffuse phenotype of gastric cancer. *E-cadherin* encodes a cell-cell adhesion molecule. It was shown to be inactivated (by germline mutation) in familial gastric cancers, playing a role in gastric cancer susceptibility (Guilford *et al*, 1998). Loss of functional *E-cadherin* induces the scattered morphology and invasive behavior of diffuse tumors, by loss of cell aggregation and gain of cell motility (Handschuh *et al*, 1999; Jawhari *et al*, 1999). Machado *et al* (1999) showed that *E-cadherin* inactivating mutations constitute the

genetic basis for the phenotypic divergence of mixed gastric carcinomas (tumors with both intestinal and diffuse components).

2.1.5.2. Oncogenes

Several genes encoding tyrosine-kinase receptors are altered in gastric carcinoma, leading to an increased growth potential and abnormal proliferation of tumor cells.
K-RAS, an example of a tyrosine-kinase receptor, is often overexpressed and mutated in gastric cancer (Motojima *et al*, 1994; Hongyo *et al*, 1995) and has been shown to be involved in intestinal-type cancers, but not in the diffuse-type cancers (Tahara *et al*, 1996a).

Another tyrosine-kinase receptor, for keratinocyte growth factor, is *K-SAM* (kato-III stomach amplified) that was shown to be amplified not only in the Kato-III cell line, but also in other gastric cancer cell lines (Hattori *et al*, 1990). This oncogene was shown to be amplified only in gastric cancers of the diffuse histologic type (Tsujimoto *et al*, 1997).

Amplifications of the *C-MET* gene, located at 7q and encoding the receptor for hepatocyte growth factor, have been observed by different authors (Kuniyasu *et al*, 1993; Seruca *et al*, 1995b; Nessling *et al*, 1998) in both histologic types (Tahara *et al*, 1996a; Carneiro, 1997) and have been related with gastric carcinoma invasion (Kuniyasu *et al*, 1993; Tsugawa *et al*, 1998; Nakajima *et al*, 1999).

TPR-MET rearrangements, leading to the activation of *C-MET*, are often detected in gastric carcinomas of intestinal-type (Soman *et al*, 1991). Recently, Yu *et al* (2000) showed that this rearrangement, occurring in 47% of the gastric carcinomas analyzed, can be already detected in normal mucosas of the relatives of patients with gastric cancer, suggesting that it may be an early event in gastric cancer development.

Amplification of the tyrosine-kinase receptor *C-ERBB2* gene, located at 17q, is often associated with intestinal-type carcinomas (David *et al*, 1992; Tahara *et al*, 1996a; Tsujimoto *et al*, 1997; Nakajima *et al*, 1999) and has been related with a more aggressive behavior of the tumors (David *et al*, 1992) and late stages of progression (Tsugawa *et al*, 1998).

Activation of oncogenes, by amplification and mutation, is also observed in transcription factor genes like *C-MYC* (located at 8q) which has been described to be amplified in gastric cancer (Kim *et al*, 1993; Tatsuta *et al*, 1994; Seruca *et al*, 1995b).

Recently, the *AIB1* gene (nuclear receptor co-activator gene) was proposed as the target gene of the amplification observed at 20q in gastric cancer (Nessling *et al*, 1998). Sakakura *et al* (2000) observed amplification of *AIB1* in 7% of the cases analyzed and overexpression in 40% of the cases. Not all the cases with overexpression showed amplification, suggesting that other mechanisms of activation besides amplification appear to be involved. However, amplification and overexpression were associated with the occurrence of lymph nodes and liver metastases and therefore with a poor prognosis.

Besides receptors and transcription factors, growth factors and cytokines are also frequently overexpressed in gastric cancer (Tahara, 1995; Tahara *et al*, 1996a).

Overexpression of the growth factor *IGF2* (Insuline-like growth factor 2) was found in gastric carcinoma (Wu *et al*, 1997b; Shiraishi *et al*, 1998). Wu *et al* (1997b) showed , in a series of 70 gastric carcinomas, that loss of imprinting (LOI) of *IGF2*, leading to overexpression of the gene, occurred in 34,5% of the informative cases. LOI was more frequent in diffuse than in intestinal carcinomas.

Another growth factor with a growth inhibitor effect in normal cells, is *TGFβ1* (transforming growth factor β1). Although the *TGFβ1* gene normally inhibits cell-cycle progression, it has an oncogenic role whenever its pathway is altered. Enhanced expression of *TGFβ1* in gastric cancer cells usually occurs in diffuse-type cancers (Tahara *et al*, 1996a). Recently, Ebert *et al* (2000) showed that *TGFβ1* expression is detectable, not only in gastric cancer patients, but also in gastric mucosas of first-degree relatives but not in healthy volunteers, suggesting that *TGFβ1* is a good marker for detection of individuals with an increased risk of developing gastric cancer.

2.1.5.3. Metastasis-related genes

Gene products frequently described as having a role in metastasis development are CD44 (adhesion molecule), urokinase-type plasminogen activator (uPA) and matrix metalloproteinases (MMPs) (Tahara *et al*, 1996a; Honda *et al*, 1996; Adachi *et al*, 1998; Migita *et al*, 1999). Different variants of *CD44* abnormal transcripts are observed in intestinal and diffuse carcinomas (Yokozaki *et al*, 1994; Tahara *et al*, 1996a).

Expression of *MMP* and *uPA* is more pronounced in the intestinal-type than in the diffuse-type cancers (Migita *et al*, 1999).

NM23, a candidate metastasis suppressor gene implicated in several human cancers like melanomas, breast, colon and hepatocellular carcinomas (Hennessy *et al*, 1991; Florenes *et al*, 1992; Nakayama *et al*, 1992; Wang *et al*, 1993), has also been implicated in gastric cancer progression, although with controversial data. Some authors observed a significant down-regulation of *NM23* gene in invasive and metastatic gastric cancer cases (Kodera *et al*, 1994; Hsu *et al*, 1999), whereas others observed that *NM23* expression was positively correlated with aggressive tumor growth and poor prognosis (Muller *et al*, 1998; Wang *et al*, 1998).

2.1.5.4. Microsatellite instability

The microsatellite instability (MSI^+) phenotype is the consequence of a deficiency in the mismatch repair mechanisms of cells, leading to the non-correction of replication errors (RER) occurring during DNA replication (Ionov *et al*, 1993.). MSI^+ phenotype is observed in 23-30% of cases of gastric carcinoma, usually of the intestinal histologic type, and is associated with a significantly better prognosis when compared to microsatellite stable (MSS) tumors (Seruca *et al*, 1995c; Dos Santos *et al*, 1996). Sequences with mononucleotide or dinucleotide repeats can lead to slippage of the DNA polymerase during replication, which, if not corrected, will cause deletions and/or insertions in these regions. This type of alterations can occur throughout the genome but also in coding regions that harbor repeat sequences. Target genes (with repeat sequences in their coding region) like, *TGFβRII*, *IGF IIR*, and *BAX* genes are frequently mutated in gastric carcinoma (Souza *et al*, 1996; Wang *et al*, 1997; Oliveira *et al*, 1998; Iacopetta *et al*, 1999; Yamamoto *et al*, 1999). Association of mutations in *TGFβRII*, *IGF IIR*, and *BAX* genes with the intestinal histotype, low prevalence of lymph node metastases and diminished penetration of the gastric wall, respectively, suggested that the good prognosis of these tumors may be mediated by mutations in the target genes (Oliveira *et al*, 1998).

A summary of the most frequently observed genetic alterations in the two main types of gastric cancer is shown in Table 1.

Table 1. Genetic alterations in the two main types of gastric carcinoma [a]

Intestinal carcinoma	Diffuse carcinoma
#20 (polysomy)	#20 (polysomy)
# 1p; 3p; 6q; 13q; 18q (LOH)	# 1p; 3p; 6q; 13q; 18q (LOH)
# 5q, 17p (LOH) *b*	
TP53 (LOH/mutation)	*TP53* (LOH/mutation)
APC (LOH)	
FHIT (LOH)	*FHIT* (LOH)
SAMD4 (LOH)	
	E-Cadherin (mutation)
RAS (overexpression/mutation)	
	K-SAM (amplification)
C-MET (amplification)	*C-MET* (amplification)
TPR-MET rearrangement	
C-ERBB2 (amplification)	
EGF (overexpression)	
AIB1 (amplification/overexpression)	
	IGF2 (overexpression)
	TGFβ (overexpression)
CD44 (abnormal transcripts)	*CD44* (abnormal transcripts)
u-PA (overexpression)	
MMP (overexpression)	
NM23 (loss)	*NM23* (loss)
MSI^+	

[a] Only chromosomes and genes with information regarding their role in the different histologic types are presented in this table.

[b] Based in Seruca *et al* (1995a).

3. Chromosome 6 and cancer

Chromosome 6 is one of the most frequently rearranged chromosomes in human neoplasia (Teyssier and Ferre, 1992). Non-random deletions of the long arm of chromosome 6 have been reported, by cytogenetics or LOH studies, in many different types of neoplasia (Stenman *et al*, 1989; Millikin *et al*, 1991; Devilee *et al*, 1991; Morita *et al*, 1991; Foulkes *et al*, 1993; Taguchi *et al*, 1993; Griffin *et al*, 1994; Menasce *et al*, 1994a; Merlo *et al*, 1994; Mitra *et al*, 1994; Tibiletti *et al*, 1997; Seruca *et al*, 1995a; Cooney *et al*, 1996; Tahara *et al*, 1996b; Huang *et al*, 1999), suggesting that the long arm of chromosome 6 might harbor one or more TSGs with a role in tumorigenesis.

3.1. Minimal regions of deletion of chromosome 6 in different types of cancer

Deletions of the long arm of chromosome 6 are observed both in hematological malignancies and in solid neoplasias (see Figure 1).

Deletions of the 6q arm are found in 20-60% of lymphomas (Gaidano *et al*, 1992; Offit *et al*, 1993; Parsa *et al*, 1994) and in at least 30% of leukemias (Finn *et al*, 1998; Merup *et al*, 1998; Hatta *et al*, 1999).

The most frequently described deletions in lymphomas are located in the following regions: (1) 6q21 (Gaidano *et al*, 1992; Offit *et al*, 1993; Menasce *et al*, 1994a; Offit *et al*, 1994; Schlegelberger *et al*, 1994; Guan *et al*, 1996; Sherratt *et al*, 1997; Zhang *et al*, 2000) (2) 6q23 (Offit *et al*, 1993; Menasce *et al*, 1994a; Zhang Y *et al*, 1997), and (3) 6q25-q27 (Gaidano *et al*, 1992; Offit *et al*, 1993; Parsa *et al*, 1994). These regions actually cover most of the long arm of chromosome 6, from 6q21 to 6qter.

In leukemias, deletions of 6q usually involve the region around 6q21 (Menasce *et al*, 1994a; Menasce *et al*, 1994b; Gerard *et al*, 1997; Wong *et al*, 1997; Finn *et al*, 1998; Merup *et al*, 1998; Takeuchi *et al*, 1998; Hatta *et al*, 1999; Zhang *et al*, 2000). Sherratt *et al* (1997) have narrowed the 6q21 region to a 2 Mb interval by FISH analysis using YAC probes, in a panel of 32 cases of lymphomas and leukemias (14 non-Hodgkin lymphomas - NHL and 18 acute lymphoid leukemias - ALL).

Deletions of 6q are found in several types of solid tumors, including melanomas, mesotheliomas and epithelial tumors.

In malignant melanomas chromosome 6 abnormalities, leading to loss of 6q segments distal to 6q11-q13, have been described as non-random events (Trent *et al*, 1989; Guan *et al*, 1998). LOH studies showed that deletions of 6q occur in a high percentage of cases, between 30% and 40% (Millikin *et al*, 1991; Walker *et al*, 1994; Walker *et al*, 1995; Healy *et al*, 1996). Approximately 40% of malignant mesotheliomas also show deletions of the long arm of chromosome 6 (Taguchi *et al*, 1993). Several minimal regions of deletion have been defined within the segment 6q14-q25 by microsatellite analysis in a series of 46 malignant mesotheliomas (Bell *et al*, 1997).

Abnormalities in the long arm of chromosome 6 also play an important role in tumors of epithelial origin, like breast (Devilee *et al*, 1991; Orphanos *et al*, 1995a),

ovarian (Cliby *et al*, 1993; Foulkes *et al*, 1993), cervix (Atkin *et al*, 1990; Mitra *et al*, 1994), endometrial (Tibiletti *et al*, 1997), renal cell (Morita *et al*, 1991; Thrash-Bingham *et al*, 1995), prostate (Cooney *et al*, 1996), salivary gland (Stenman *et al*, 1989; Jin *et al*, 1994), pancreatic (Griffin *et al*, 1994; Abe *et al*, 1999), hepatocellular (Huang *et al*, 1999) and lung carcinomas (Merlo *et al*, 1994).

In breast carcinoma, 6q deletions are already present in early tumor stages (Chappell *et al*, 1997), as well as in benign lesions. Cin *et al* (1996) identified a 6q deletion in a case of benign fibrocystic disease. These results suggest that 6q deletions are an early genetic event in breast cancer development. The same was suggested for ovarian carcinoma where deletion of 6q segments are detected in early stage tumors (Orphanos *et al*, 1995b; Tibiletti *et al*, 1996), before the development of aneuploidy (Abeln *et al*, 1994).

Smallest regions of overlap (SROs) were defined in several types of carcinoma, pointing to the involvement of different genes in their development.

In breast carcinoma, different groups defined different SROs within the segments 6q13-q21 (Orphanos *et al*, 1995a; Noviello *et al*, 1996; Sheng *et al*, 1996; Theile *et al*, 1996), 6q23-q25 (Orphanos *et al*, 1995a; Fujii *et al*, 1996; Noviello *et al*, 1996; Sheng *et al*, 1996; Theile *et al*, 1996) and 6q25-q27 (Orphanos *et al*, 1995a; Noviello *et al*, 1996; Theile *et al*, 1996; Chapell *et al*, 1997). As also described in haematological malignancies, most regions along 6q arm, from 6q13-q21 to 6qter, are covered. The same regions are involved in ovarian tumors: one proximal to 6q21 (Cliby *et al*, 1993; Orphanos *et al*, 1995b), one within 6q21-q25 (Wan *et al*, 1994; Orphanos *et al*, 1995b; Colitti *et al*, 1998; Shridlar *et al*, 1999) and another within 6q26-q27 (Cliby *et al*, 1993; Foulkes *et al*, 1993; Lastowska *et al*, 1994; Wan *et al*, 1994; Orphanos *et al*, 1995b; Rodabaugh *et al*, 1995; Shridhar *et al*, 1999). In a detailed deletion mapping of 32 ovarian carcinomas, Saito *et al* (1996) further narrowed the 6q27 region to a segment of 300 kb, a size that allows the search for candidate genes in the region.

In other types of tumors this large region of deletion, from 6q21-q27, was also described, namely in hepatocellular, renal cell, prostate, salivary gland and pancreatic carcinomas.

LOH at 6q26-q27 is found in approximately 60% of hepatocellular carcinomas (De Souza *et al*, 1995a). This deletion was detected in 2 out of 3 hepatocellular adenomas of the analyzed series of 36 tumors, suggesting that this loss is an early event in the etiology of tumors of the liver. In a study of 25 hepatocellular carcinomas Huang *et al* (2000) defined a 2 cM SRO in a different region of the long arm of chromosome 6, at 6q14. This 2 cM size allows the search for candidate genes implicated in hepatocellular carcinomas.

The region implicated in renal cell carcinoma is 6q27, with LOH described in almost 40% of informative cases (Morita *et al*, 1991).

In prostate cancer the region involved is 6q14-21, with a percentage of LOH of 33% (Cooney *et al*, 1996).

Queimado *et al* (1998), in a LOH analysis, defined two SROs in salivary gland carcinomas, one interstitial at 6q21-q23.3 (1,5 Mb) and another distal at 6q27 (approximately 2 Mb).

In pancreatic carcinoma, Abe *et al* (1999), defined 3 SROs, one at 6q21 (500 kb), one at 6q23-q24 (7 cM) and the other at 6q26 (13 cM), and covered the former region (500 kb) with overlapping BAC clones in order to start isolating and characterizing candidate TSGs.

A summary of the deletions of the long arm of chromosome 6 described above for all different types of neoplasias is depicted in Figure 1. Three regions of the long arm of chromosome 6 are mainly implicated, two interstitial - 6q16-q21 and 6q23-q24 – and one terminal – 6q26-q27. Some genes located in these regions are considered tumor suppressor candidates (to be discussed below).

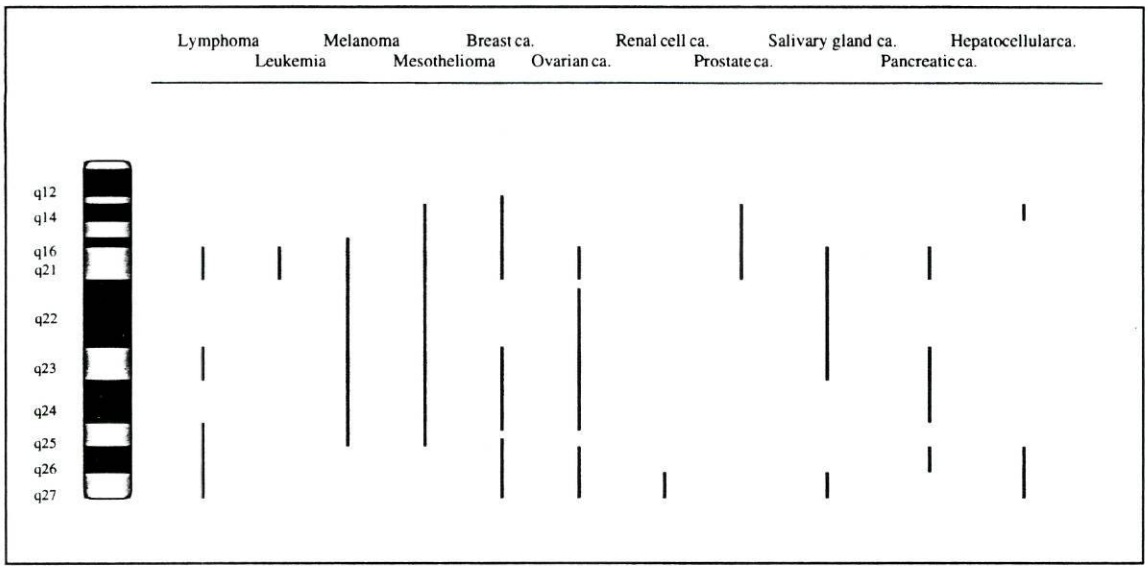


Figure 1. Schematic representation of minimal regions of deletion in different types of human cancer. Ca., carcinoma.

3.2. Chromosome 6 transfer

Microcell-mediated chromosome transfer studies support the idea of existence of tumor suppressor genes on chromosome 6. Trent *et al* (1990) showed that introduction of an intact chromosome 6 into melanoma cells suppressed their ability to form tumors in nude mice. Robertson *et al* (1996) suggested that suppression of tumorigenicity of melanoma cell lines after introduction of chromosome 6 is correlated with an increased chromosome 6 dosage, rather than with the presence of the transferred normal copy of this chromosome, since no preferential loss of the transferred chromosome was observed in the clones that reacquired the tumorigenic phenotype. This group also showed that deletion of the 6q22-q24 segment in the donor chromosome did not prevent the suppression of tumorigenicity. Welch *et al* (1994) observed that the introduction of chromosome 6 into metastatic melanoma cell lines did suppress the formation of metastases but not tumorigenicity, suggesting the existence of a gene with a metastasis-suppressing role on chromosome 6. This gene appears to inhibit metastases through regulation of cellular motility, since hybrids cells (with chromosome 6 introduced) were found to be less motile than the parental cells (You *et al*, 1995). Recently, a melanoma metastasis-suppressor *locus* was suggested to map at region 6q16.3-q23 as the introduction of a chromosome 6 with a deletion in this region, into a metastatic

melanoma cell line, did not suppress the formation of metastases in athymic mice (Miele *et al*, 2000).

Suppression of the tumorigenic phenotype following chromosome 6 transfer was also obtained by Negrini *et al* (1994) in breast cancer cell lines. Furthermore, these authors verified that revertant clones, that reacquired the tumorigenic phenotype, presented deletions in two region(s) - 6q21-q23 and/or 6q26-q27. A defined chromosome 6q fragment (6q23.3-q25) was shown to be sufficient to suppress the malignant phenotype when introduced in a breast cancer cell line (Theile *et al*, 1996).

Also in ovarian carcinoma, it was shown that the introduction of a normal chromosome 6 into tumor cell lines leads to the suppression of the tumorigenic phenotype (Wan *et al*, 1999). The region previously shown to be sufficient to suppress the malignant phenotype in breast cancer (6q23.3-q25) (Theile *et al*, 1996) had the same effect in ovarian carcinoma, since whenever a 2 cM fragment at this region was deleted in the donor chromosome 6, ovarian tumor cells regained tumorigenicity (Wan *et al*, 1999).

Some authors contend that genes at chromosome 6 appear to have a role in cell senescence. Human cells in culture have a limited life span. After a number of cell generations, cells become senescent, showing morphological changes and ceasing proliferation. In contrast, many human tumors overcome senescence and cells grow continuously, becoming immortal. Hubbard-Smith *et al* (1992) showed that immortallized fibroblasts had deletions of the long arm of chromosome 6, distal to 6q21, suggesting that 6q harbors one or more genes related to senescence, which when inactivated, lead to cell immortalization. The introduction of a human chromosome 6 into immortal human fibroblasts, transformed by SV40 virus (Sandhu *et al*, 1994) and into BK virus transformed mouse cells (Gualandi *et al*, 1994) induced senescence of these cells; the fact that cells that kept the immortal phenotype, had deletions in 6q21-q22 and 6q25 (Gualandi *et al*, 1994), reinforces the hypothesis that these regions harbor senescence genes. Recently, two senescence genes, *SEN6* (Banga *et al*, 1997) and *SEN6A* (Sandhu *et al*, 1996), have been mapped to 6q26-q27 and 6q14-q21, respectively.

3.3. Candidate tumor suppressor genes on 6q

Several genes mapping to the long arm of chromosome 6 were already isolated and implicated as putative TSGs in different cancer models.

In acute leukemias two genes on 6q were described as being partner genes in translocations with the *ALL1/MLL* gene at 11q23. One *ALL* fusion partner is *AF6*, located at 6q27, described in the translocation t(6;11)(q27;q23), found in acute myeloid leukemias (AML) (Prasad *et al*, 1993) and the other is *AF6q21*, involved in acute myeloid lymphoblastic leukemia (MLL) in the translocation t(6;11)(q21;q23) (Hillion *et al*, 1997). The protein AF6 is associated with the cytoskeleton of the cell (Prasad *et al*, 1993), being one of the components of tight junctions in epithelial cells and of cell-cell adhesions in non-epithelial cells, and participating in the regulation of cell-cell contacts (Yamamoto *et al*, 1997). AF6q21 protein shows strong similarities with forkhead proteins, that have a DNA binding domain and transcription and regulation properties (Hillion *et al*, 1997). Medema *et al* (2000) demonstrated that overexpression of forkhead transcription factors causes growth suppression in transformed cell lines. They conclude that these proteins are involved in cell-cycle regulation and that inactivation of these proteins is an important step in oncogenic transformation. In a B-cell ALL cell line, with a t(6;12)(q23;p13), a previously unknown gene at chromosome 6 was described – *STL* (six-twelve leukemia gene), that was fused to *ETV6* at 12p13 (Suto *et al*, 1997). The role of the fusion protein ETV6/STL is still not clear but one hypothesis is the disruption of the normal function of both transcripts (Suto *et al*, 1997). Another gene that was recently described to play a role in human B-cell leukemia is *TLX* (human homologue to *Drosophila* tailess gene – *tlx*), mapping at 6q21 (Jackson *et al*, 1998). *TLX* appears to be a transcription regulator. Its putative role in the pathogenesis of hematological malignancies has to be clarified by mutation analysis in patients carrying 6q deletions.

Also mapping at 6q21, the gene *CCNC*, encoding human cyclin C, was suggested to be a tumor suppressor, since it is lost in a high percentage (>90%) of ALL cases with 6q21 deletions (Li *et al*, 1996a). Cyclin C regulates the kinase catalytic domain of cdk8, having a role in cell-cycle progression, and it was suggested that an alternative spliced form of cyclin C protein (truncated protein) found in avian cells, might function as a negative regulator of the cyclinC/cdk8 complex activity (Li *et al*, 1996b).

As mentioned previously (see in section 3.2 of this introduction), malignant melanoma suppression is induced by the introduction of a chromosome 6 into tumorigenic cell lines, implicating the existence on this chromosome of TSGs/melanoma suppressor genes (Trent *et al*, 1990; Welch *et al*, 1994). One candidate gene is *SOD2*, located at 6q25, a region frequently lost in melanoma (Figure 1) (Millikin *et al*, 1991). It encodes the antioxidant enzyme manganese superoxide dismutase (MnSOD). It was shown by Church *et al* (1993) that introduction of MnSOD cDNA into melanoma cell lines, altered the phenotype of the cells in culture and suppressed their ability to form colonies in soft agar and tumors in nude mice, having the same effect as when introducing an entire chromosome 6. Bravard *et al* (1992) suggested that *SOD2* was a new type of TSG, where activity is dependent on the gene dosage and is directly related with cell differentiation. Nonetheless, there are conflicting observations, where no changes in gene dosage were observed, but a reduction in *SOD2* expression was detected in the tumor. A change in the signal transduction machinery related to metals (Mn, Fe) deficiency, may have limited the binding of transcription factors to DNA, leading to a reduction in MnSOD (Borrello *et al*, 1993). Contradicting these data, it was shown that transfection of a functional *SOD2* into metastatic melanoma cell lines, did not change the metastatic potential of the cells nor their tumorigenicity (Miele *et al*, 1995). More recently, Alvarez *et al* (1998) showed that metastatic melanoma cells that lost their metastatic potential after introduction of chromosome 6, presented increased basal expression of Cu/ZnSOD and MnSOD when compared to their metastatic counterpart. With these conflicting results, the role of *SOD2* in malignant transformation is still not clear.

Ray *et al* (1996), using a subtraction technique, comparing a chromosome 6 suppressed melanoma cell line with the parental cell line, isolated a series of uniquely expressed genes in the suppressed cell line. One of these genes was designated as *AIM1* (absent in melanoma) and is located at 6q21, a region already shown to be frequently deleted in melanomas (Figure 1) (Millikin *et al*, 1991). Characterization of the *AIM1* gene and its major transcripts revealed that AIM1 protein belongs to the βγ–crystallin superfamily (Ray *et al*, 1997). β- and γ- crystallins are expressed in elongating lens fiber cells that undergo large changes in cytoskeletal architecture and composition, and thus appear to play a role in cell morphology. In addition, *AIM1* contains regions with similarities to actin-binding domains, which suggests that it is also involved in the management of cell morphology. The disruption of the *AIM1* gene would lead to lack of

mediators of normal cell morphology contributing to cells to become anchorage-independent and invasive, and thereby increasing the tumorigenic and metastatic potential of cells (Ray *et al*, 1997).

In breast cancer, several genes from the long arm of chromosome 6 have been pinpointed as candidate tumor suppressor genes, namely the estrogen receptor gene (*ER*), mapped at 6q25.1, one of the regions frequently lost in breast carcinoma as previously mentioned (Orphanos *et al*, 1995a; Noviello *et al*, 1996; Fugii *et al*, 1996). The relationship between loss of expression of the *ER* with allelic loss in breast cancer is not very clear. Iwase *et al* (1995) showed that the *ER* was lost only in 19% of informative cases suggesting that allele loss does not play an important role in the lack of ER function in breast cancer tissues. However, Chappell *et al* (1997) found a higher percentage of LOH of the *ER locus* (33% and 46% of informative cases, in cases of ductal carcinoma *in situ* and invasive carcinomas, respectively). Magdelénat *et al* (1994) showed that major variations in ER expression were independent of the number of copies of the *ER* gene. However, they did observe that the average ER expression was lower in tumors with deletions in 6q compared to tumors without deletion, suggesting a mere additional role of gene dosage. Another candidate TSG is the type II insulin-like growth factor receptor/cation-independent mannose 6-phosphate receptor gene (*M6P/IGF IIR*) mapped at 6q26-q27, a region also frequently lost in breast cancer (Orphanos *et al*, 1995a; Noviello *et al*, 1996; Theile *et al*, 1996; Chappell *et al*, 1997). This gene has been shown to harbor inactivating point mutations in the remaining allele in breast carcinomas (Hankins *et al*, 1996) as well as in hepatocellular carcinomas (De Souza *et al*, 1995b), supporting a role of *M6P/IGFIIR* as tumor suppressor in malignant transformation, since its function of degrading IGF2 (mitogen) and activating TGFβ (growth inhibitor) is hampered in these tumors.

Recently a new candidate tumor suppressor was described in ovarian carcinomas (Abdollahi *et al*, 1997) and breast carcinomas (Bilanges *et al*, 1999) – *LOT1/ZAC* (Lost on transformation/zinc finger protein; induction of apoptosis; control of cell cycle progression). This gene resides at 6q25, a region commonly lost in both types of tumors (Foulkes *et al*, 1993; Orphanos *et al*, 1995a). Bilanges *et al* (1999) showed that despite the lack of mutations in the remaining allele, *ZAC* expression is lost or down-regulated due to aberrant gene methylation, hampering its antiproliferative properties (induction of apoptosis and G1 arrest).

The receptor-type protein tyrosine phosphatase (*PTPRK*) maps at 6q22.2-q22.3 and is a negative regulator of tyrosine kinases acting through modulation of phosphorylation (Zhang *et al*, 1998). It plays a role in TGFβ-dependent inhibition of cell proliferation (Yang *et al*, 1996). Fuchs *et al* (1996) showed that PTPRK forms a complex with catenins and colocalizes with these proteins at adherens junctions, suggesting a role in the regulation of cell contact and adhesion. Because of its biochemical properties and chromosomal location (often deleted in human cancer) *PTPRK* is a good candidate for TSG in this region.

Human histone deacetylase 2 (*HDAC2*), recently localized to chromosome 6q21 (Betz *et al*, 1998), belongs to a group of genes that act as modulators of chromatin structure, leading to repression of transcription. This gene is a good TSG candidate, since it was shown that transcriptional repression of a subset of E2F binding-site containing genes occurs through interaction of the RB protein with a histone deacetylase (Brehm *et al*, 1998). Again, the chromosomal region where it maps is frequently deleted in human cancer (as shown above; Figure 1).

In Figure 2, the cytogenetic position on long arm of chromosome 6 of the described genes is shown.

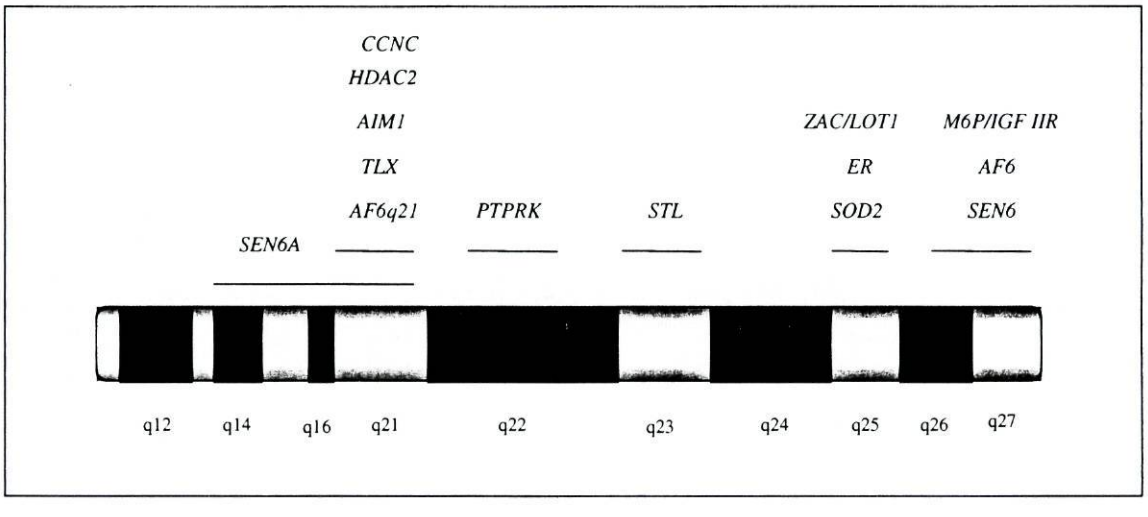


Figure 2. Localization of candidate tumor suppressor genes on long arm of chromosome 6.

Summing up, all genes described seem to play a role in transformation of human cells. Mutational and functional studies will be necessary to clarify their role in human carcinogenesis.

3.4. Chromosome 6 and gastric carcinoma

Chromosome 6 is frequently altered in gastric carcinoma (as already shown in point 2 of this introduction). Rearrangements of the long arm of chromosome 6 resulting in the loss of the segment distal to 6q21 were described in 27-45% of cases of gastric carcinoma (Ochi *et al*, 1986; Seruca *et al*, 1993; Panani *et al*, 1995).

Loss of heterozygosity studies showed that 6q deletions occur in 29-53% of cases (Seruca *et al*, 1995a; Schneider *et al*, 1995; Gleeson *et al*, 1997). This loss is found in all histologic types of gastric carcinoma (Seruca *et al*, 1995a; Gleeson *et al*, 1997) and in both early and advanced tumors (Seruca *et al*, 1995a).

Two smallest regions of overlap (SROs) of heterozygous deletions were delimited (Queimado *et al*, 1995). One region, which was heterozygously lost in 50% informative cases, was mapped at 6q16.3-q23.1 and spans 15 cM. The other region, heterozygously lost in 37% of informative cases, was mapped distally to 6q23-q24 and spans more than 30 cM.

To our knowledge, none of the genes mapping at 6q (see Figure 2) were implicated in gastric cancer development leading to the question, which gene(s) are located at this regions at 6q and which is their role in gastric cancer development. To address this question, a positional cloning approach was undertaken. The first step was to delimit further the two SROs by detailed deletion mapping and the second step was to start looking for candidate genes by physical analysis of the region.

4. Positional cloning

Positional cloning is the process by which genes involved in genetic disorders can be identified, as a result of genetic analysis and without previous knowledge on the function of the genes. This process has several steps: (1) Localization of the disease gene *locus* to a particular region of a chromosome, through the identification of chromosomal abnormalities or by genetic linkage analysis in families segregating for the disease; (2) Detailed genetic mapping and physical mapping of the region; (3)

YAC/BAC contig construction; (4) Identification of transcripts (or expressed sequences); (5) Mutation analysis of candidate genes (Collins, 1992; Monaco, 1994; Collins, 1995).

4.1. Strategies to isolate genes

There are several techniques available for gene identification (Parrish and Nelson, 1993). A general description of these techniques will be given with more emphasis on the differential screening techniques, one of which was used in the work done in this thesis.

Mainly there are two different approaches to search for genes, on one hand methods that use cloned DNA, and on the other hand methods that use non-cloned DNA.

In the former group (methods that use cloned DNA), there are strategies that are dependent on expression and strategies that do not depend on expression. Expression-dependent techniques are: (a) screening of cDNA libraries using YAC inserts as probes (Elvin *et al*, 1990) and (b) cDNA selection methods where cDNA fragments are hybridized with immobilized YAC DNA and the hybrids recovered by PCR (Parimoo *et al*, 1991; Lovett *et al*, 1991). Expression-independent techniques are: (a) cross-species sequence homology, where DNA fragments are hybridized to Southern blots of genomic DNA isolated from multiple species (Zoo blots) (Monaco *et al*, 1986); (b) identification of CpG islands within DNA fragments, since unmethylated CpG-rich regions are frequently associated with expressed sequences (Bird, 1986); (c) exon trapping (Duyk *et al*, 1990; Buckler *et al*, 1991) where DNA fragments are cloned into a vector, transfected into mammalian cells, allowing the screening for exons existing in the insert due to the presence of splice sites in the DNA insert; and (d) screening for splice sites using splice site consensus sequences as probes, that hybridize with immobilized DNA (Melmer and Buchwald, 1992).

Still within the former group of methods, that use cloned DNA, is the sequencing analysis of genomic DNA (YACs, BACs, P1 clones), where the obtained sequences are analyzed by sophisticated software developed to predict exon-intron boundaries.

In the second group of approaches (methods that use non-cloned DNA) fit all the methods based on differential expression: RNA fingerprinting (Welsh *et al*, 1992), differential display (Liang and Pardee, 1992), serial analysis of gene expression (SAGE)

(Velculescu *et al*, 1995) and subtraction-based techniques (Sagerström *et al*, 1997; Diatchenko *et al*, 1996; Lisitsyn *et al*, 1993). These techniques will be discussed below.

Another strategy to find genes included in the second group of approaches is the use of expression sequence tags (ESTs). ESTs result from random sequencing of cDNA clones. Typically, ESTs are 300-500 bp long, a sufficient size to establish the identity of the expressed gene (Venter, 1993). There are currently over 1,5 million human ESTs in the publicly available databases of ESTs (dbESTs) provided by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/dbEST/index.html). Identification of thousands of genes expressed in the brain was successfully achieved using this approach (Adams *et al*, 1992). Also, new members of gene families can be identified by the analysis of ESTs data (Adams *et al*, 1992; Almeida *et al*, 1997; Amado *et al*, 1999).

4.1.1. Differential expression-based approaches

In this section attention will be drawn to strategies based on the differential expression of genes in different cell populations (from different tissues or in different developmental times). Some of the positive as well as negative aspects will be referred for each strategy, although all the techniques described below have been successful in the identification of genes.

A simple and fast approach is RNA fingerprinting (Welsh *et al*, 1992) which compares paired total RNA populations, from which cDNA is produced and amplified using arbitrary primers. The PCR products are run side by side in a sequencing gel. Dissimilar bands that correspond to differentially expressed genes, can be selected and purified from the gel for further analysis. As examples of successful application of RNA fingerprinting are the identification of differentially regulated RNAs in mink lung (Mv1Lu) cells growth arrested by TGFβ1 (Ralph *et al*, 1993) and the isolation of human transcribed sequences from human YAC inserts (Still *et al*, 1997).

A conceptually similar technique is differential display (DD) (Liang and Pardee, 1992), where mRNA populations are compared after PCR amplification. Amplification is done with a poly-d(T) and arbitrary primers and separation of the resulting cDNAs is done by denaturing polyacrylamide gel electrophoresis. Since its first description (Liang and Pardee, 1992), several improvements have been published (Liang *et al*, 1993; Liang and Pardee, 1995; Chen and Peck, 1996; Matz *et al*, 1997; Bosch *et al*, 2000) and successful experiments have been achieved. Examples of genes isolated by DD are the

cytokeratin endo A and α-subunit of the mithocondrial F1 ATP synthase genes expressed during mouse pre-implantation development (Zimmermann and Schultz, 1994). The identification of differentially expressed mRNAs in human melanoma cell lines with different metastatic capacity was also possible with this technique (Van Groningen *et al*, 1995).

Drawbacks of these techniques are the high number of false positives, the questioned ability to identify both abundant, and rare transcripts and the time-consuming verification process that demands large amounts of RNA (Wan *et al*, 1996; Matz and Lukyanov, 1998).

Subtractive hybridization is a powerful technique that allows the isolation of differentially expressed sequences of two cell populations, isolating mRNAs present in one type but absent from the other type of cells. Subtraction techniques are based on the nucleic acid re-association technology (complementary nucleic acids reannealing) (Sagerström *et al*, 1997). The basic steps of a subtraction protocol are: (1) the hybridization of the nucleic acid from the cells from which one wants to isolate differentially expressed sequences (tracer) with excess complementary nucleic acid from the cells which, in principle, do not have the sequences of interest (driver); and (2) the removal of driver-tracer hybrids (common sequences) and unhybridized driver (Sagerström *et al*, 1997). After this subtraction, the tracer population is enriched for tracer specific sequences (target sequences) and depleted in common sequences to the tracer and driver populations. The enriched pool of target sequences is cloned and analyzed. In classical approaches, several rounds of subtraction must be performed in order to ensure that all common sequences are depleted. Following each hybridization step, non-specific target sequences need to be physically removed. Thus, the technique becomes time-consuming and laborious. Despite this drawback, several differentially expressed cDNA clones have been isolated by means of subtractive hybridization (Sagerström *et al*, 1997). Examples are cDNAs encoding specific T-cell membrane-associated proteins (Hedrick *et al*, 1984), myoblasts-specific cDNA clones (Davis *et al*, 1987) and the gene *WAF1* (El-Deiry *et al*, 1993), a mediator of p53 tumor suppression.

New PCR-based subtraction approaches have been developed in order to accelerate the technical procedure and to increase the sensitivity of this kind of approach.

One of this new methods is suppression subtractive hybridization (SSH). This method, developed by Diatchenko *et al* (1996) is based on the suppression PCR

technique and combines normalization (same abundance of cDNAs within the target population) and subtraction (exclusion of common sequences between target and driver populations), in a single procedure. This method eliminates the intermediate step of physical removal of non-specific target cDNAs. The resulting subtracted cDNA mixture can be used to generate differentially expressed or tissue-specific cDNA libraries and can be used directly as a hybridization probe for screening recombinant DNA libraries for identification of tissue-specific sequences from a particular chromosome (Diatchenko *et al*, 1996; Diatchenko *et al*, 1999). The application of the SSH method has led to the identification of 27 genes differentially expressed in an estrogen receptor-positive breast carcinoma cell line, when compared with an estrogen receptor negative breast carcinoma cell line (Kuang *et al*, 1998). Girard *et al* (1999) showed that this technique is useful for cloning of differentially expressed genes in very small samples, such is the case of high endothelial venule endothelial cells (HEVECs) when purified. Subtraction of control and iron-loaded astrocytoma cells, using SSH, led to the identification of genes regulated by cellular iron availability at the mRNA level (Ye and Connor, 2000).

Another PCR-based subtraction technique is representational difference analysis (RDA) (Lisitsyn *et al*, 1993). Here, DNA fragments that are present in only one of two nearly identical genomes are cloned (Lisitsyn *et al*, 1993). The same protocol can also be adapted to clone differentially expressed cDNAs (Hubank and Schatz, 1994). RDA, like SSH, does not require physical separation of specific and non-specific sequences, but still requires multiple rounds of subtraction. Some examples of successful application of this technique are the detection of homozygous deletions (Schutte *et al*, 1995; Zeschnigk *et al*, 1999) and amplified sequences (Simons *et al*, 1997) in cancer, isolation of new genomic markers (Baldocchi *et al*, 1996), and identification of novel genes with cancer-specific expression (Gress *et al*, 1997).

Serial analysis of gene expression (SAGE) is a technique that allows a rapid and detailed analysis of thousands of transcripts (Velculescu *et al*, 1995). It is based on two principles. First, a short nucleotide sequence tag (9-10 bp) is enough to identify a transcript. Second, concatenation of short sequence tags (a few hundred tags) allows a serial analysis of the transcripts by sequencing of multiple tags within the same clone. This technique provides both qualitative and quantitative data on gene expression

(Velculescu *et al*, 1995). SAGE has already been applied to study gene expression profiles in normal and cancer cells (Zhang L *et al*, 1997).

Because most of the methods used in gene identification have inherent disadvantages as well as unique advantages, many studies combine two or more techniques in order to improve the final results.

Suzuki *et al* (1996) used the method ESD, that combines equalization of cDNAs with subtractive hybridization and differential display, to isolate specific genes from embryonal carcinoma after retinoic acid treatment. Also, Lee and Welch (1997) combined subtractive hybridization with differential display to identify highly expressed genes in metastasis-suppressed melanoma cell lines. The integrated procedure for gene identification (IPGI) method combines the ESTs information, subtraction hybridization, suppression PCR, SAGE and sequencing for genome-wide gene analysis (Wang and Rowley, 1998). Combining SSH with the emerging technology of cDNA microarrays, allows the rapid identification of differential expressed genes, by efficiently screening subtracted cDNA libraries spotted on microarrays with cDNA probes derived from different cell lines (Yang *et al*, 1999).

In this thesis we tried to isolate and map at the same time cDNAs from a restricted region of the genome (at long arm of chromosome 6). For that we analyzed cDNA from yeast containing YACs from the region by means of suppression subtractive hybridization (SSH).

Chromosome 6 is frequently altered in gastric carcinoma. Both cytogenetic and molecular analysis (LOH studies) showed that specific segments of the long arm of chromosome 6 are lost in this type of neoplasia, regardless of its histologic type or stage. Two smallest regions of overlap (SRO), have been defined in a panel of sporadic tumors. One is interstitial, between 6q16.3-q21 -> 6q22.3-q23, lost in 50% of informative cases, and the other is distal to 6q23-q24, at 6q26-q27, lost in 37% of informative cases. All these results suggest the existence of tumor suppressor gene(s) on 6q that might play a role in gastric carcinogenesis. Taking this into account, the main goal of this work was to study the alterations of this chromosome in gastric carcinoma in more detail with special attention to the regions pinpointed previously.

Specific aims:

- Detailed deletion mapping of the interstitial SRO that spans a genetic distance of 15 cM. By means of microsatellite analysis, comparing tumor tissue from sporadic primary tumors with correspondent normal mucosa, we aimed at restricting this region to a segment that would allow us to start looking for genes putatively involved in gastric carcinoma development.

- Microsatellite and FISH analysis of the 6q content of tumors xenografted in nude mice and respective derived cell lines, and comparison of the data with the primary tumors. In xenografts and/or cell lines the problem of admixed normal tissue does not occur. Moreover, these xenografts and/or cell lines represent an unlimited source of material. Xenografts and cell lines thus represent a very valuable tool for future studies.

- Construction of a transcription map. After delimiting the interstitial SRO, we aimed to isolate cDNAs from this region. This was achieved by using a subtraction protocol (Suppression subtractive hybridization).

MATERIAL AND METHODS

The material and methods used in the different studies will be briefly presented according to the issues raised in the Aims section. Some of the methods used are described in detail in Papers I to III.

1. Detailed deletion mapping of the interstitial SRO

1.1. Mapping *locus* D6S32 by Southern analysis of YACs and PACs spanning the segment 6q16.3-q23.3

Preparation of p3B10 probe

- p3B10 insert is cloned in the plasmid vector pBR322. We digested the plasmid with the restriction enzyme *Hind*III and purified the band which corresponded to the insert, using the Qiaquick Gel extraction kit (Quiagen). The insert was cloned into a pBlueScript vector for sequencing.

Preparation of YAC DNA

- YACs mapping at chromosome 6 and chromosome 3 (as negative control) were selected from the twelfth release from the Human Physical Mapping Project at the Whitehead Institute database/MIT Center for Genome Research. YACs were selected that contained STS markers described to be in the region of *locus* D6S32. These included YACs from contigs WC6.14 and WC6.15. All YACs used throughout this thesis were kindly made available to us by Dr. E.F.P.M Schoenmakers, University of Leuven (Belgium). After culturing the yeast strains in AHC^- medium (25 µg/ml ampicilin) for 36-48h at 30 °C, spheroplasts were obtained by digestion of the yeast cell wall as described by Green and Olson (1990). YAC DNA was isolated by standard salt-chloroform extraction procedures (Müllenbach *et al*, 1989).

Southern analysis

- DNA samples (YACs and human DNA) were digested with restriction enzyme *Msp*I, separated on 0.8% agarose gel by electrophoresis and transferred to nylon membranes by alkaline blotting (Southern, 1975). The insert of p3B10 was labeled with [α-^{32}P] dCTP by the random primer extension method (Feinberg and Vogelstein, 1982), using the Multiprime DNA labeling system (RPN1601z-Amersham). The blots were

prehybridized for more than 2 hours at 65 °C in a buffer consisting of 0.5M $NaHPO_4$ (pH 7.2), 1mM EDTA and 7% SDS, followed by hybridization at 65 °C overnight with the probe. Membranes were washed at 65 °C with solutions of increasing stringency (2x SSC/0.1% SDS; 1x SSC/0.1% SDS; 0.3x SSC/0.1% SDS; 0.1x SSC/01% SDS). Membranes were exposed to X-ray film (SuperRX from Fuji or X-OMAT-AR from Kodak) at –70 °C with an intensifying screen for 1-3 days.

Screening of PAC DNA libraries

- The RPCI-1 and RPCI-6 human PAC libraries (Children's Hospital Oakland Research Institute; http:// www chori.org) were screened with the insert of p3B10 (D6S32) according to standard procedures.

1.2. Detailed deletion mapping of the interstitial SRO by microsatellite analysis (Paper I)

Material

- 60 primary gastric carcinomas and respective non-neoplastic gastric mucosas were selected. The material was collected immediately after surgery, frozen in liquid nitrogen and stored at –70 °C until DNA extraction. The tumors were classified according to Láuren's classification – 30 intestinal carcinomas, 15 diffuse carcinomas and 15 atypical carcinomas.

DNA extraction

- High molecular weight DNA was isolated using the salt-chloroform extraction method (Müllenbach *et al*, 1989). When paraffin blocks of diffuse tumors were available, areas rich in tumor cells were microdissected and DNA was isolated by phenol-chloroform extraction (Jen *et al*, 1994).

Microsatellite analysis

- 21 microsatellite markers mapping at 6q (19 within 6q16.3-q23.3; 1 proximal at 6q14-q16.2 and one distal at 6q27) were selected from the human genetic map of

Gyapay *et al* (1994) and from the Cedar database accessible at http: // cedar.genetics. soton.ac.uk/pub.

- 3 microsatellite markers mapping at 3p21.3 (Kok *et al*, 2000) and 3 microsatellite markers mapping at 17p12-p13 were also selected and used in the analysis of a subset of the carcinomas.

- Polymerase chain reaction (PCR) was performed using standard techniques. Briefly, after an initial denaturation step at 94 °C for 5 min, amplification was for 30 cycles with denaturation at 94 °C for 0.5 min, annealing at 53 to 60 °C (depending on the primer set) and extension at 72 °C for 1.5 min. Following the last cycle an additional extension step at 72 °C for 5 min was performed. PCR products were labeled with [α^{32}P] dCTP during amplification, denatured at 94 °C for 5 min, in a solution containing 95% formamide, separated by electrophoresis in a denaturing 6% polyacrylamide and visualized through autoradiography. Evaluation of allelic imbalances was done by densitometry on an Ultrascan XL LKB densitometer using Gel Scan XL 2.1 software (Pharmacia LKB).

Analysis of the location of AIM1 gene relative to the 2-cM SRO

- YACs (870b3, 776a5, 798g12, 911f5, 785d2, 951b2, 826f8, 903h8) from the chromosomal regions that spans *loci* D6S278 and D6S302 were screened for the *AIM1* gene by PCR. All YACs were selected from the contig WC6.14 (Whitehead Institute/MIT Center for Genome Research).

- Primers specific for the *AIM1* gene were designed: Forward 5'-TCACATTATCCTCAACACTGTC-3' (nt 4842-4863); Reverse 5'CAGTCTTGAAAT CTTGATATAAC-3' (nt 5202-5224), giving rise to a product of 382 bp.

- After an initial denaturation step at 94 °C for 3 min, amplification was for 30 cycles with denaturation at 94 °C for 0.5 min, annealing at 60 °C and extension at 72 °C for 1.5 min. Following the last cycle, an additional extension step at 72 °C for 3 min was performed.

2. Analysis of 6q status in xenografts and cell lines derived from primary gastric carcinomas (Paper II)

Material

- 6 primary gastric carcinomas (CE195, CE199, CE202, CE220, CE226 and CE233) were heterotransplanted in nude mice (N: NIH(s)II – nu/nu), and 3 cell lines (GP195, GP202 and GP220) were established from three of the xenografts. The cell lines were grown in RPMI 1640, supplemented with 4 mg/ml L-glutamin, 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin.

DNA extraction

- High molecular weight DNA was isolated from frozen material of the xenografts and from pellets of the cell lines, using the salt-chloroform extraction method (Müllenbach *et al*, 1989).

Microsatellite analysis

- From the panel of 21 microsatellite markers mapping at 6q, only 13 were used in this analysis. Two more distal markers mapping at 6q27 were added to the analysis, making a total of 15 markers in 6q. All analyses were carried out at least *in duplo*. PCR was performed as described above (point 2 of this section). Signal intensities were determined either by densitometry on an Ultrascan XL LKB densitometer using Gel Scan XL 2.1 software (Pharmacia LKB), or on a phosphor-imaging system (STORM 860, Molecular Dynamics).

FISH analysis

- FISH analysis of the three cell lines (GP195, GP202, GP220) was performed according to routine procedures with the following probes: a) a Bio-11-dUTP-labeled chromosome 6-specific library (wcp6); b) a digoxigenin-11-dUTP labeled-chromosome 6 centromeric probe (p308), in combination with Bio-11-dUTP-labeled CEPH YAC 870b3 (6q16.3-q21), c) CEPH YACs 801c2 (6q21-q23.3) and 820f9 (6q27) CEPH YACs labeled with digoxigenin-11-dUTP and Bio-11-dUTP, respectively, or d) a digoxigenin-11-dUTP labeled-chromosome 6 centromeric probe (p308), in combination with Bio-11-dUTP-labeled CEPH YAC 820f9 (6q27). Chromosomes were counterstained with DAPI. For each analysis 25 metaphases were analyzed.

3. Construction of a transcription map of the 2-cM SRO (Paper III)

Material

- A YAC contig covering the SRO region (6q16.3-q23.3) was assembled by selecting CEPH YACs from the YAC contig WC6.14, from the Whitehead Institute database. YACs were selected when they had unambiguous hits with STS markers used in the microsatellite analysis (Point 2 of this Material and methods section).

Microsatellite analysis of the YAC contig

- PCR amplification YAC DNA was with the same microsatellite markers that were used in the detailed deletion mapping (Point 2 of this Material and methods section), in order to check their physical position relative to the markers.

Pulsed-Field gel Electrophoresis

- The size of the YACs was analyzed by Pulsed-Field Gel Electrophoresis (PFGE) (Sambrook *et al*, 1989) in order to check the size of the YACs. Briefly, after culturing in AHC^- medium (25 µg/ml ampicilin) for 36-48h at 30 ºC, the yeast cells were pelleted, washed once, and mixed with liquidified 1% low-melting point agarose. Solid agarose plugs were made with an approximate volume of 100µl. Lysis of yeast cell wall and of the cells was done by incubating the plugs with zymolease (0.4 mg/ml) and with proteinase K (0.2 mg/ml), respectively. The agarose plugs were then washed and loaded into a 1% agarose gel. Electrophoresis was at 10 °C for 46h (20 to 120-second pulses for 18h; 120-second constant pulses for 10h and 120 to 140 second-pulses for 18h) at a constant voltage (120v) with perpendicularly orientated electric fields. Visualization of the separated DNAs in the gel was done by ethidium bromide staining. DNA was transferred to nylon membranes by acid/alkaline blotting. Cot-1 DNA was labeled with [α-^{32}P] dCTP by the random primer extension method (Feinberg and Vogelstein, 1982), using the Multiprime DNA labeling system (RPN1601z-Amersham). Hybridization and washing conditions were the same as used in point 1 of this section. Membranes were exposed to autoradiography (SuperRX from Fuji) and developed after 1-3 days.

PolyA$^+$ RNA extraction

- Total RNA from four different CEPH YACs (y776a5, y785d2, y826f8 and y946d3) was isolated according to the RNAzol™ B procedure (Campro Scientific).

- Poly A$^+$ RNA was obtained using the mRNA purification kit from Amersham Pharmacia Biotech.

cDNA synthesis

- Double stranded cDNA was synthesized from 2 µg of poly A$^+$ RNA using AMV reverse transcriptase and Taq-DNA polymerase, included in the Clontech's PCR-Select™ cDNA subtraction kit, following the instructions of the supplier.

cDNA subtraction

- Supressive cDNA subtraction was done according to the instructions of the Clontech's PCR-Select™ cDNA Subtraction Kit. Briefly, double stranded tester and driver cDNAs were digested with *Rsa*I restriction enzyme. Digested tester cDNA was divided in two tubes and different adaptors were ligated (a different adaptor in each tube). A first hybridization was performed between the driver cDNA and each adaptor-ligated tester cDNA, separately. A second hybridization was done with more driver cDNA together with the two samples from the first hybridization. After hybridization, differentially expressed cDNAs were selectively amplified by PCR. A modification in the procedure was introduced at this point - diluted cDNAs (subtracted and unsubtracted samples) were further diluted 50x before primary PCR. Efficiencies of *Rsa*I-digestions and adaptor ligations were checked as recommended in the kit. A summarized scheme of the technique is showed in Figure 3.

- Subtraction efficiency was tested by PCR of the yeast housekeeping gene ZWF1 (encoding glucose-6-phosphate dehydrogenase). Primer sequences were: 5'-GGATTCCAGAGGCTTACGAG-3' (forward), 5'-GGGTGCTTTTCGGGCATAAC-3' (reverse). Amplification was for 30 cycles with denaturation at 94 °C for 0.5 min, annealing at 58 °C for 0.5 min, and extension at 72 °C for 2 min. The initial denaturation step at 94 °C and the final extension at 72 °C lasted for 3 min. PCR was performed on the unsubtracted and on the subtracted non-cloned cDNA.

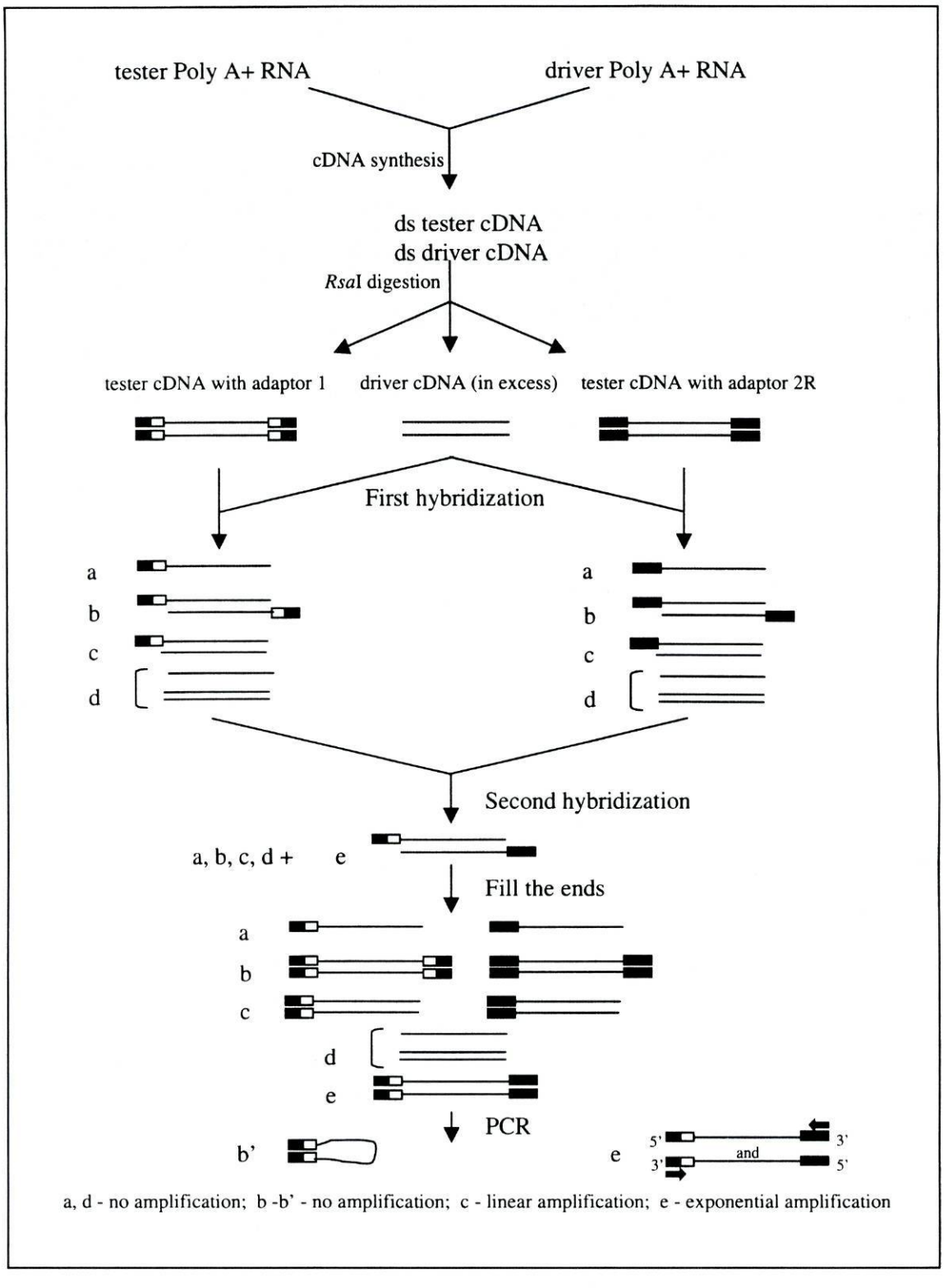


Figure 3. Schematic representation of the suppression subtractive hybridization (SSH) technique.

cDNA library construction

- PCR products, resulting from the subtraction, were subcloned into the pCR™II vector using the TA Cloning kit (Invitrogen). The transformed bacteria were plated on selective plates and white colonies were selected randomly. Three cDNA libraries were constructed – y776a5-specific cDNAs library (192 clones); y785d2-specific cDNAs library (192 clones) and y826f8-specific cDNAs library (384 clones).

Analysis of the cDNA libraries

- For further analysis of the cDNA libraries we dot-blotted PCR products to nylon membranes. Briefly, the inserts of the selected colonies were amplified by touch–PCR. The PCR was carried out in a 25-µl reaction volume containing 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM $MgCl_2$, 200 mM of each deoxynucleotide triphosphate, 0.4 mM of each primer (M13 forward 5'-GTAAAACGACGGCCAG-3'; M13 reverse 5'-CAGGAAACAGCTATGAC-3'), and 0.5 U of Taq DNA polymerase (Pharmacia). Amplification was for 30 cycles with denaturation at 94°C for 0.5 min, annealing at 50°C for 0.5 min, and extension at 72°C for 1.5 min. The initial denaturation step at 94°C and the final extension step at 72°C lasted for 3 min. 7µl of the PCR product was analyzed on a 2% agarose gel. The PCR products were denatured by the addition of an equal volume of 0.5M NaOH, and 3-µl aliquots were dot-blotted on nylon membranes (Hybond-N^+; Amersham Pharmacia Biotech). Four identical copies of each filter were made. DNA was cross-linked to the membranes by incubation at 80 °C for 2 hours.

- The membranes were hybridized with both uncloned subtracted cDNA pools. Hybridization and washing steps were done as described above (point 1 of this section). cDNA clones that hybridized predominantly with the cDNA pool from which they originated, were considered specific for that cDNA pool.

Southern analysis

- Genomic DNA blots (containing human placenta DNA and DNA from the tester and driver YACs, from YACs overlapping tester and driver YACs, and from a distal 6q YAC as negative control) were prepared by Southern blotting (as described above in point 1 of this section). Probes and Southern membranes were competed with 10 µg and 15 µg human Cot-1 DNA, respectively, before hybridization. Hybridization and washing steps were done as described above (point 1 of this section).

Sequencing analysis

- Sequencing of selected clones was done on a ABI Prism ™377 DNA Sequencer (Perkin Elmer) using M13 primers from the pCR™ vector. The cDNA sequences were compared to the available databases using BLAST search (http://www.ncbi.nlm.nih.gov /blast/blast.cgi).

Northern analysis

- Expression analysis of positive cDNA clones was evaluated by hybridization with commercial available multiple-tissue Northern (MTN) blots from Clontech (#7759-1- spleen, thymus, prostate, testis, ovary, small intestine, colon (no mucosa), peripheral blood leukocyte; #7767-1- stomach, thyroid, spinal cord, lymph node, trachea, adrenal gland, bone marrow; #7780-1- brain, heart, skeletal muscle, colon (no mucosa), thymus, spleen, kidney liver, small intestine, placenta, lung, peripheral blood leukocyte).

- The hybridization procedure was done following the supplier's instructions. Briefly, cDNA probes were [α-^{32}P] dCTP labeled by random primer labeling (Multiprime DNA labeling system-RPN1601z-Amersham). MTN blots were pre-hybridized with ExpressHyb™ solution for 60 min at 68 °C and hybridized with the labeled cDNA probe overnight at 68 °C. Membranes were first washed at room temperature for 30 min with 2x SSC/0.05% SDS and then at 50°C with 0.1x SSC/0.1% SDS. Membranes were exposed to X-ray film (X-OMAT-AR from Kodak) at –70 °C with an intensifying screen and autoradiographs developed after 3-10 days.

RESULTS

1. Detailed deletion mapping of the interstitial SRO

The interstitial SRO (6q16.3-q23.1) previously reported by our group (Queimado *et al*, 1995) is defined by *locus* D6S32 (RFLP marker p3B10) that was lost in 50% of informative cases of the sporadic gastric carcinomas analyzed. The flanking markers of this SRO are D6S268 and *ARGI*. New markers in this region were selected for further delimitation of the deleted 6q segment. Within the new panel of markers we tried to localize the exact position of *locus* D6S32.

1.1. Mapping *locus* D6S32 by Southern analysis of YACs and PACs spanning the segment 6q16.3-q23.3

The genetic position of D6S32, previously assigned to 6q22.3 (Rao *et al*, 1993) is currently deposited in the databases as being at 6q23.3 (http://cedar.genetics.soton.ac.uk/pub/ chrom6/map.html; http://gdbwww.gdb.org).

YACs from the Whitehead Institute database (http://www-genome.wi.mit.edu/) that map to the long arm of chromosome 6, in the region where D6S32 is supposed to map (6q23.3), were selected as described in the Materials and methods section (Figure 4).

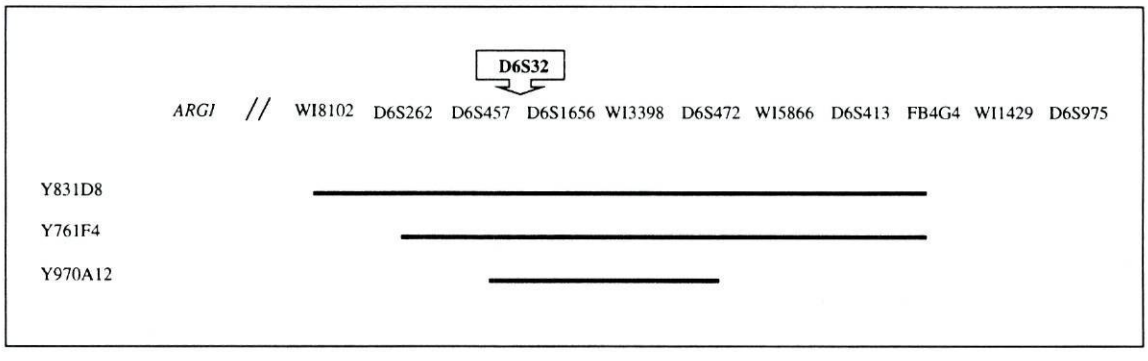


Figure 4. Schematic representation of the presumed location of D6S32 relative to the markers (http://cedar.genetics.soton.ac.uk/pub/chrom6/ map.html) and to the YACs that map to this region (YAC contig WC6.15).

When p3B10 (*locus* D6S32) was hybridized to DNA of the YACs immobilized on the Southern blot membranes, no signal was detected in any of the them. A specific hybridization signal was obtained only in the DNA of human placenta and DNA of a panel of 5 gastric mucosas used as positive controls of the hybridization (Figure 5).

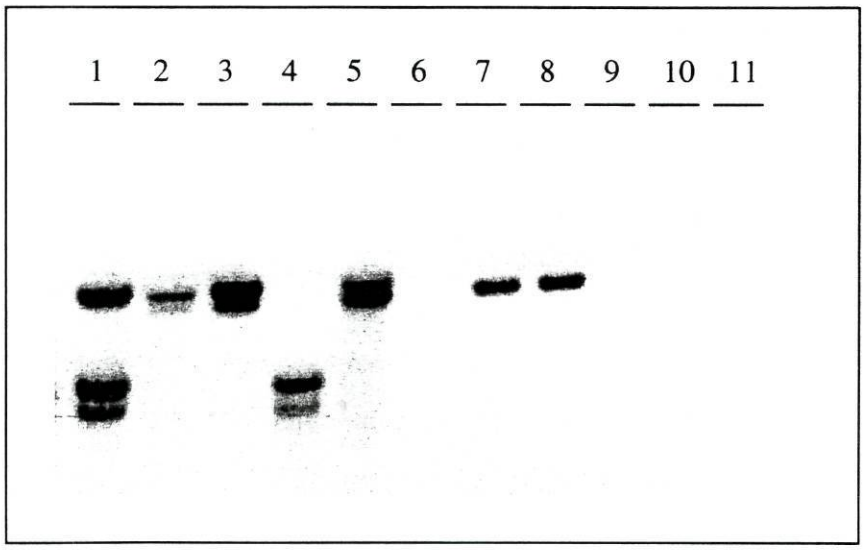


Figure 5. Southern analysis of YAC DNA and human DNA (human placenta and gastric mucosas). Lane1, mucosa 184; lane 2, mucosa 191; lane 3, mucosa 192; lane 4, mucosa 159; lane 5, mucosa 182; lane 6, λ*Hind*III; lane 7, human placenta 1; lane 8, human placenta 7; lane 9, Y761F4 (#6); lane 10, Y831D8 (#6); lane 11, Y743C5 (#3). Probe, p3B10 (D6S32); All samples digested with *Msp*I restriction enzyme.

The interstitial SRO was reported to map at 6q16.3-q23.1, proximally to *ARG1* (Queimado *et al*, 1995). We thus included in the analysis a YAC contig that extended from D6S278 until AFM268vh5, a segment of some 7 cM, proximal to *ARG1*. Again, no specific hybridization signals were observed in any of these YACs, that is, we did not see a band with the same length as in human DNA (positive control) (Figure 6).

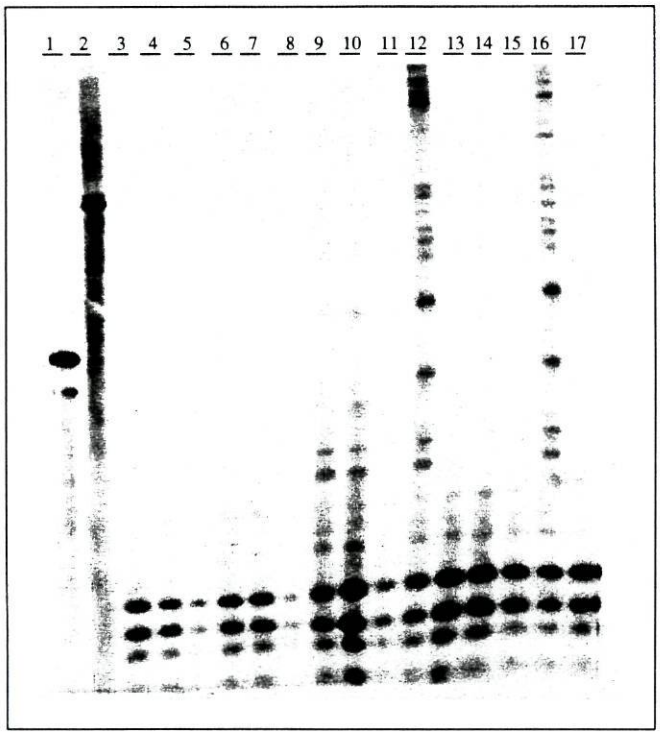


Figure 6. Southern analysis of YACs mapping at 6q (6q16.3-q23.1). Lane 1, λ*Hind*III; lane 2, human placenta 7; lane 3, y870b3; lane 4, y826f8; lane 5, y852d6; lane 6, y933c4; lane 7, y856g2; lane 8, y748b1; lane 9, y946d3; lane 10, y884b9; lane 11, y919h8; lane 12, y801c2; lane 13, y901c1; lane 14, y831d8; lane 15, y761f4; lane 16, y970a12; lane 17, y743c5 (#3). Probe, p3B10 (D6S32); Samples digested with *Msp*I.

To facilitate further search for the location of D6S32, we screened the RPCI 1 and 6 PAC libraries with p3B10 probe. The screening did not result in the selection of any PACs that contained D6S32 sequences.

In yet one other approach, the insert of p3B10 was subcloned into the vector pBlueScript, and the sequence was determined. Screening of the available databases with this sequence revealed an almost 100% identity with clone 2023J2 from the CIT-HSP library (accession nr. B64516).

1.2. Detailed deletion mapping of the interstitial SRO by microsatellite analysis (Paper I)

The interstitial SRO, defined by Queimado *et al* (1995), was flanked by the markers D6S268 (6q16.3-q21) and *ARGI* (6q22.3-q23.1), and spanned a region of 15 cM. To further delimit this region we increased the number of markers within the region 6q16.3-q23.3. We selected 19 microsatellite markers that were highly polymorphic and had a genetic distance between each other of 0 to 5 cM. In addition, we selected one proximal (6q14-q16.2) and one distal (6q27) marker (Figure 7).

We analyzed with these markers 60 primary gastric tumors. Of the 50 tumors that were informative for at least one *locus* within the region 6q16.3-q23.3, 18 (36%) showed allelic imbalance (AI). The overlap of these cases allowed us to define a SRO of 2 cM (approximately 3 Mb), flanked by D6S278 and D6S404 (Figure 8).

AI was detected in all histologic types of gastric carcinoma (10 intestinal, 4 diffuse and 4 atypical tumors) and it was observed both in early as in advanced tumors.

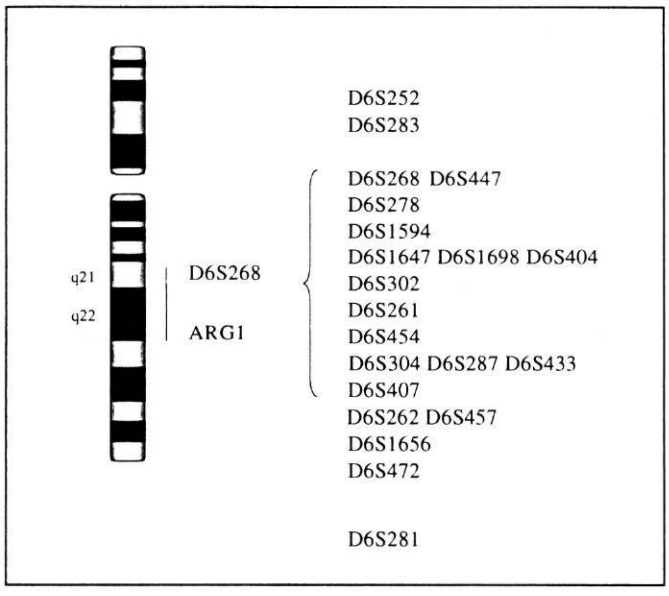


Figure 7. Schematic representation of the panel of microsatellite markers used in this study.

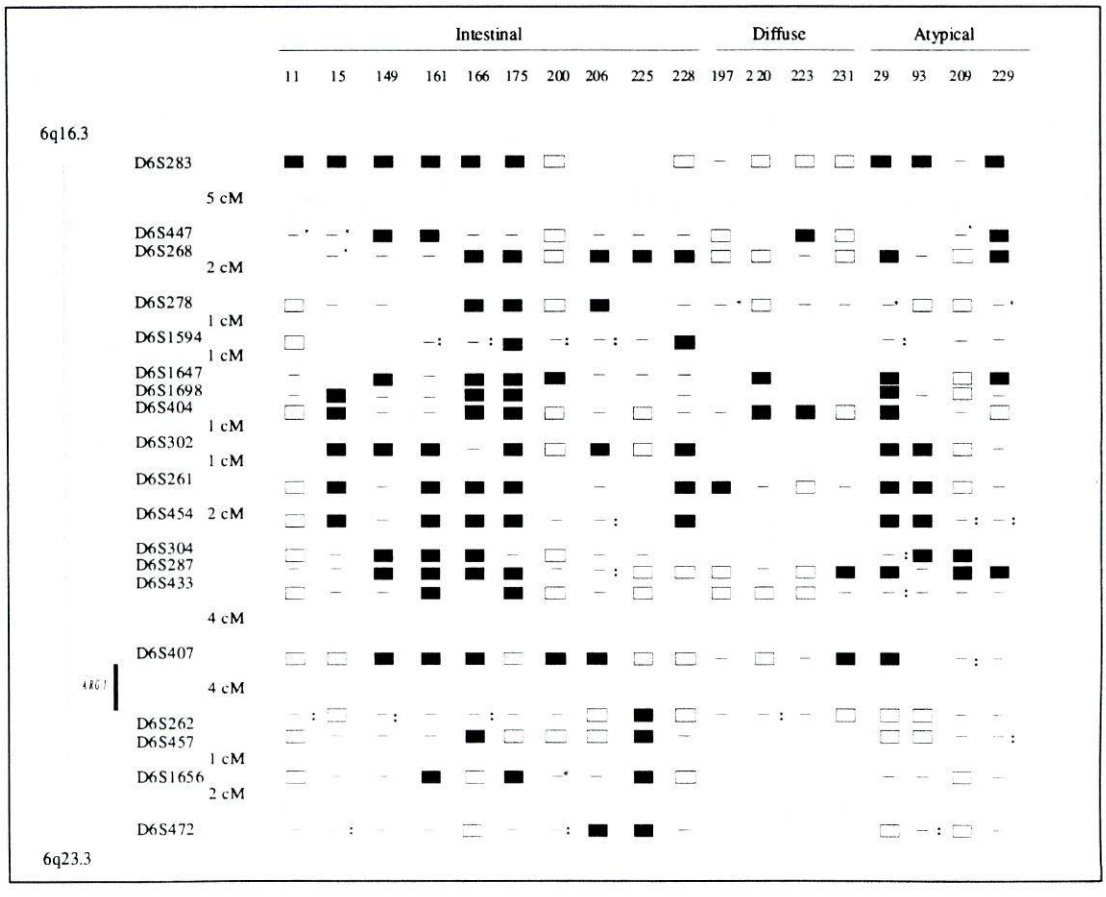


Figure 8. Patterns of allelic imbalance (AI) at 6q16.3-q23.3. ■, AI; □, retention of heterozygosity; -, not informative (homozygous); -:, not informative (alleles separated only by two base pairs); ., microsatellite instability (MIN); intestinal, tumors of intestinal histologic type; diffuse, tumors of diffuse histologic type; atypical, tumors of atypical histologic type; shadowed region, smallest region of overlap (SRO) of heterozygous deletions. The relative order of the markers was based on the genetic map of Cedar database.

Comparison of the frequency of AI at the 6q-SRO (D6S1594, D6S1647, D6S1698) with two other hotspots of heterozygous deletions in gastric cancer, namely 3p21.3 and 17p13, was done in a subset of 30 tumors. Table 2 shows summarized results of this analysis. The SRO at 6q and the 17p13 region showed comparable percentages of AI. For the 6q-SRO, the frequency of AI was 35% (8 of 23 informative cases) and for 17p13 this was 38% (8 of 21 informative cases). The frequency of AI at 3p21.3 was 15% (4 of 27 informative cases).

Table 2. Allelic imbalance (AI) analysis of 6q16.3-q23.3, 3p21.3 and 17p12-p13.

			Loci		
Cases	**Histologic type**	**Tumor stage**	**6q** [a]	**3p21.3**	**17p12-p13**
29	Atypical	Early	AI	RET	RET
228	Intestinal	Early	AI	RET	NI
11	Intestinal	Advanced	RET	AI	AI
15	Intestinal	Advanced	AI	AI	RET
149	Intestinal	Advanced	AI	RET	RET
161	Intestinal	Advanced	NI	AI	AI
166	Intestinal	Advanced	AI	RET	AI
175	Intestinal	Advanced	AI	-	AI
177	Intestinal	Advanced	RET	RET	AI
200	Intestinal	Advanced	AI	RET	RET
224	Intestinal	Advanced	RET	RET	AI
202	Atypical	Advanced	RET	RET	AI
209	Atypical	Advanced	RET	AI	AI
229	Atypical	Advanced	AI	NI	NI

AI, Allelic Imbalance; RET, Retention of Heterozygosity; NI, Not Informative; -, Not Determined. [a] Only the markers *D6S1594*, *D6S1647*, and *D6S1698*, which mapped within the SRO defined in this study, have been taken into account.

Ray *et al* (1996) identified a gene on the long arm of chromosome 6 (at 6q21) – *AIM1* (absent in melanoma) and suggested that it could have a role as a tumor suppressor gene in melanoma development. We checked if this gene mapped within the 2-cM SRO defined by us. PCR amplification of YACs 870b3, 776a5, 798g12, 911f5, 785d2, 951b2, 826f8 and 903h8, that are from a contig that overlap the SRO with primers specific for *AIM1*, showed no amplification product (Figure 9). Thus, *AIM1* does not appear to map between *loci* D6S278 and D6S404 (flanking markers of the SRO).

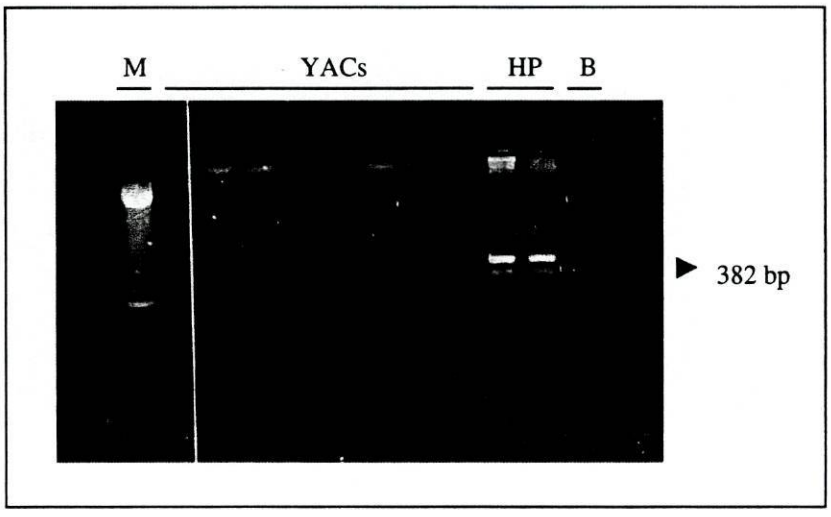


Figure 9. PCR amplification of YACs from the SRO using primers for *AIM1* gene. Lane 1, Marker (50 bp ladder); lane 2, y870b3; lane 3, y776a5; lane4, y798g12; lane 5, y911f5; lane 6, y785d2; lane 7, y951b2; lane 8, y826f8; lane 9, y903h8; lane 10 and 11, human DNA; lane 12, blank.

2. Analysis of 6q status in xenografts and cell lines derived from primary gastric carcinomas (part of the results in Paper II)

In order to compare the 6q status in the xenografts and cell lines with the primary tumors, we analyzed 6 tumors xenografted in nude mice and 3 cell lines derived from 3 of the xenografts. This analysis was done by microsatellite analysis. We selected 12 of the markers used in the analysis of primary tumors (6q16.3-q23.3), 1 proximal marker (6q14.2-16.2) and 3 distal markers mapping at 6q27, corresponding to the distal region defined by Queimado *et al* (1995). Additionally, we analyzed the 3 cell lines by FISH (chromosome 6 painting and bicolor FISH with YACs).

2.1. Microsatellite analysis

Results from the microsatellite analysis in the 6 xenografts (cases 195, 199, 202, 220, 226 and 233) and 3 cell lines (cases 195, 202 and 220) in comparison with the respective primary tumors are described below.

Figure 10 shows schematically the results obtained for cases where only the primary tumor and the xenograft were available (cases 199, 226 and 233). Cases 199 and 233 presented microsatellite instability for almost all *loci*. Case 226 showed retention in all *loci* both in the primary tumor and in the xenograft.

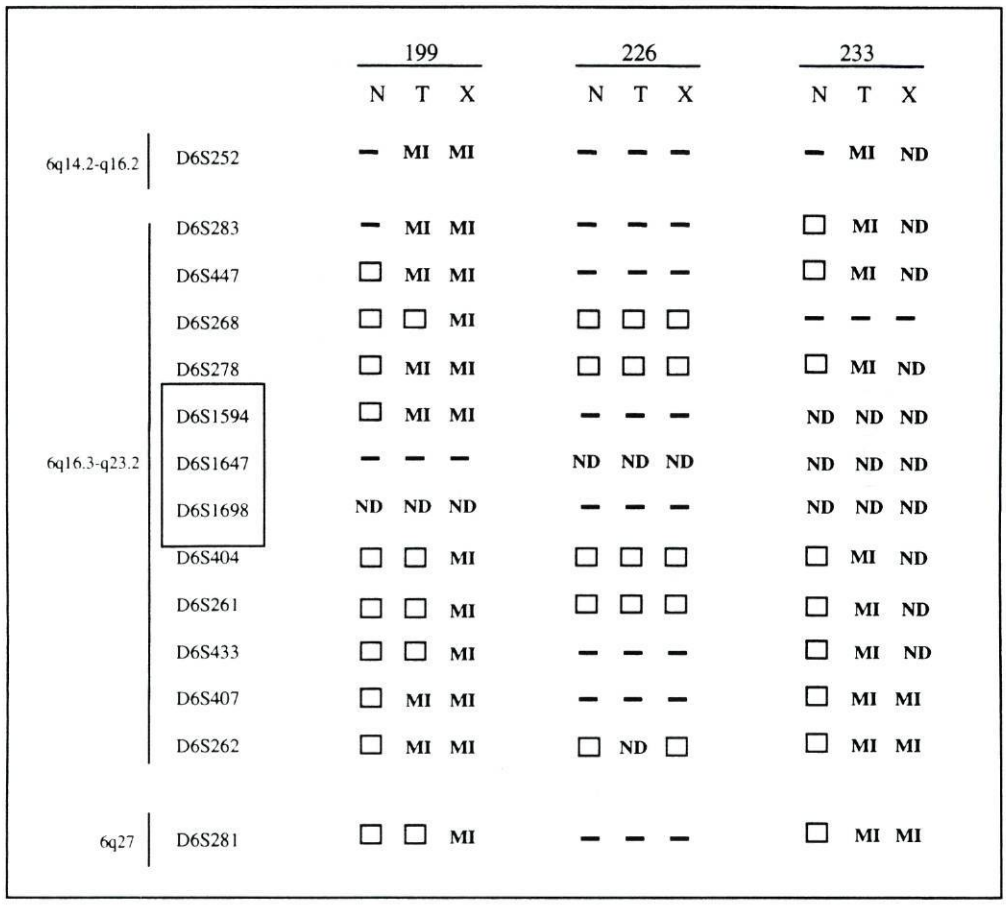


Figure 10. Microsatellite results of cases GP199, GP226 and GP233. N, normal mucosa; T, primary tumor; X, xenografted tumor. □, retention of heterozygosity;-, Not informative; **MI**, microssatellite instability; **ND**, not done. Shadowed box, 2-cM SRO.

Figure 11 shows the results obtained for the cases where cell lines were established (cases 195, 202 and 220). The xenografts and cell lines of cases 195 and 202 showed AI for all informative *loci* between D6S252 and D6S305. Complete loss of one parental allele was detected for case 195 at D6S281 and TBP, and for case 202 at D6S305, D6S281 and TBP. The xenograft and the cell line of case 220 showed AI for all

informative *loci* on chromosome 6q. No complete loss of one parental allele was detected for any marker.

For all three cases no AI was detected in the primary tumors for any of these markers. Only when tumor cells from case 220 were carefully microdissected, AI was detected for both markers that could be analyzed, namely D6S1647 and D6S404.

In the *loci* where visual inspection of the autoradiograms indicated an AI, the ratio between the alleles was quantified. The ratio's, as depicted in Figure 11 vary between 1.2 and 2.2. As shown in case 220, densitometric scanning of the autoradiograms appeared to result in lower ratios than the analysis using the phosphor-imager system.

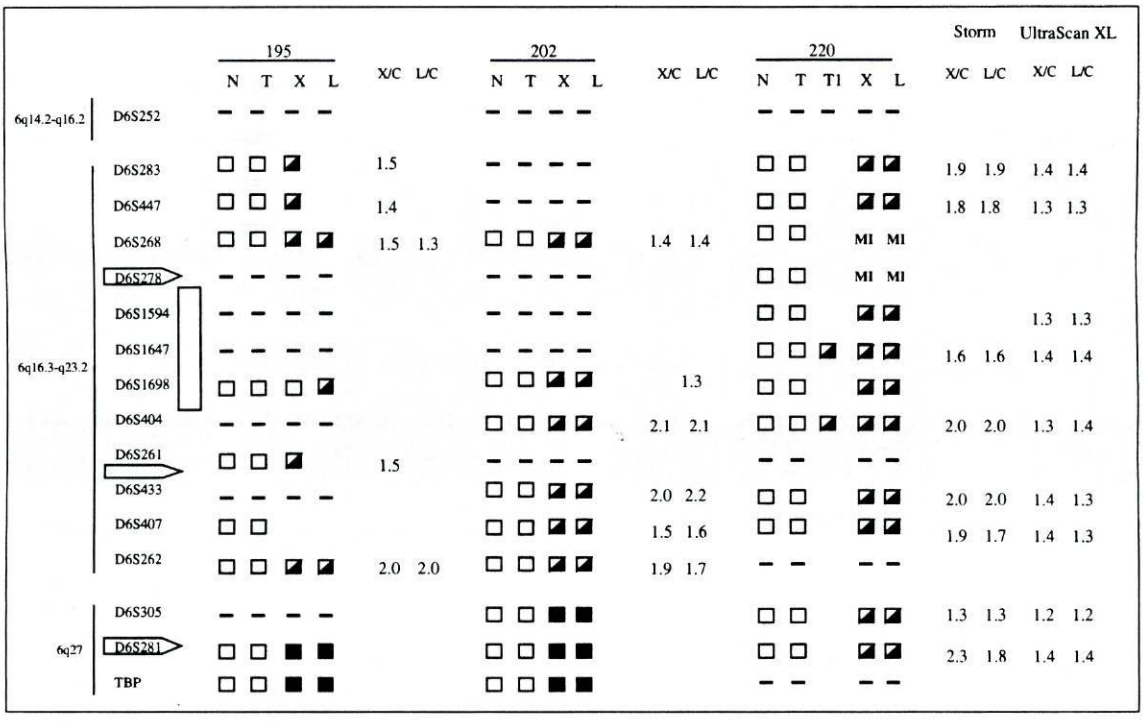


Figure 11. Microsatellite analysis of cases GP195, GP202 and GP220. N, normal mucosa; T, primary tumor; T1, microdissected primary tumor; X, xenograft in nude mice; L, cell line; X/C and L/C, allelic ratios for xenografts and cell lines, respectively. □, retention of heterozygosity; ◪, AI; ■, LOH; Dash, not informative; Blank space, not determined. Arrows, position of the YACs used in FISH analysis; shadowed box, 2-cM SRO.

2.2. FISH analysis

Three cell lines derived from the xenografts of cases 195, 202 and 220 were analyzed by FISH. All three cell lines were successively hybridized with a chromosome 6-specific library (wcp6), with y870b3 from 6q16.3-q21 in combination with a chromosome 6-specific centromeric probe, and with y820f9 from 6q27 in combination with either the centromeric probe or y801c2 from 6q21-q23.3 (Figure 12).

When hybridized with the chromosome-specific library, cell line GP195 showed two apparently normal chromosomes 6 (with respect to size as well as banding) and one derivative chromosome 6 that appeared larger than a normal chromosome 6, but nonetheless consisted entirely of chromosome 6 material (Figure 12A). The centromeric probe and the proximal YAC (y870b3) both hybridized to the same set of three chromosomes (Figure 12B). Also the middle YAC (y801c2) hybridized to three chromosomes (not shown). The distal YAC (y820f9), however, hybridized to only two of these three chromosomes (Figure 12C). All metaphases of GP202 contained one apparently normal chromosome 6 and two translocation products that consisted only in part of chromosome 6 material (Figure 12A). The centromeric probe gave a signal on two chromosomes; y870b3 gave a signal on the same two chromosomes and on one other chromosome (Figure 12B). y801c2 gave a signal on three chromosomes, and y820f9 on two of these three chromosomes (Figure 12D). Thus, like GP195, GP202 appears to contain three 6q arms, one of which is having a terminal deletion. GP220 contained one apparently normal chromosome 6 and three translocation products that consisted in part of chromosome 6 material (Figure 12A). The centromere 6-specific probe gave a signal on two chromosomes, one of which also had a signal for y870b3. In addition, this YAC gave a signal on two other chromosomes (Figure 12B). y801c2 and y820f9 also gave signals on three chromosomes (Figure 12D). GP220 clearly has three 6q arms. A schematic representation of the FISH results is shown in Figure 13.

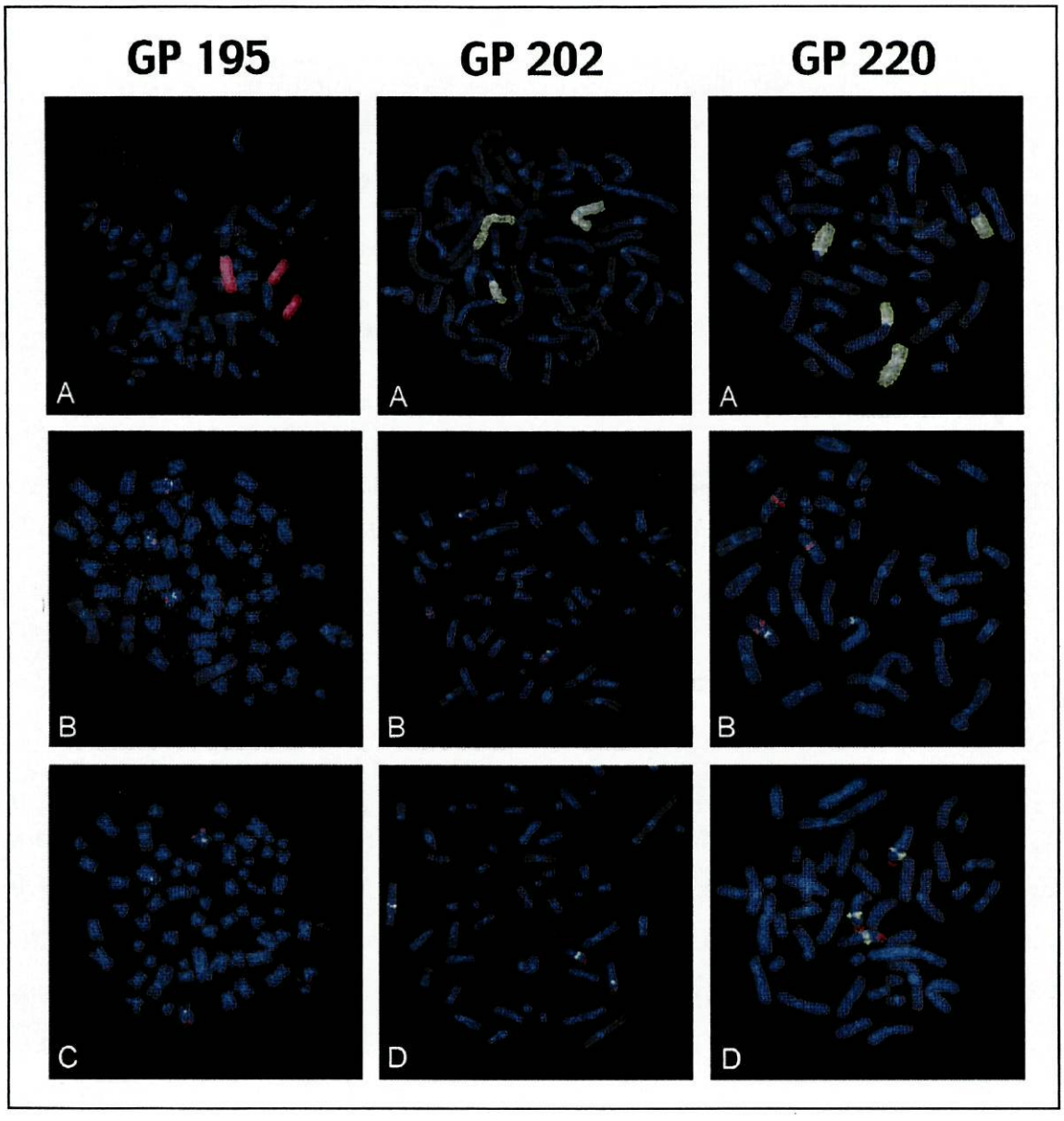


Figure 12. FISH analysis of cell lines GP195, GP202 and GP220. A, Chromosome 6 painting (probe wcp6); B, Bicolor FISH with centromere probe (green signal) and y870b3 (red signal); C, Bicolor FISH with centromere probe (green signal) and y820f9 (red signal); D, Bicolor FISH with y801c2 (green signal) and y820f9 (red signal).

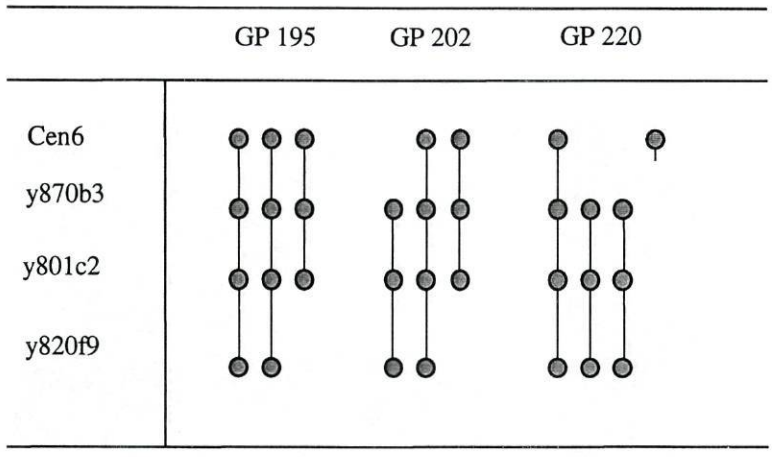


Figure 13. Schematic representation of the FISH results in the three cell lines (GP195, GP202, and GP220). Hybridization signals that appear on the same chromosome are vertically aligned.

3. Construction of a transcription map of the 2-cM SRO (part of the results in Paper III)

Assuming that human genes are being expressed in yeast, we isolated cDNAs from the 2-cM SRO by subtracting YACs that map to this region. The size and relative position of the YACs to the microsatellite markers were checked by PFGE and by PCR, respectively. Results are shown in Table 3. We selected non-overlapping contiguous YACs to obtain at the end cDNAs from the entire region. In Figure 14 the relative positions of the selected YACs within the SRO is represented. Two subtractions were performed. In the first subtraction YACs 776a5 and 785d2 were subtracted to each other (Paper III). From the forward subtraction we isolated cDNAs expressed specifically in y776a5 and in the reverse subtraction we isolated cDNAs expressed specifically in y785d2. The second subtraction was done with YACs 826f8 and 946d3. In this second subtraction, we were only interested in the cDNAs expressed specifically in y826f8 (forward subtraction), because y946d3 is outside the SRO (Figure 14).

Table 3. Size and markers content of the YACs mapping within the 2-cM SRO [a].

YACs	Size (kb)	D6S278	D6S1594	WI1240	WI3490	D6S1647	D6S1698	D6S404
776a5	990	+	+	+	-	-	-	-
798g12	910	-	-	+	-	-	-	-
911f5	1320	-	-	-	+	-	-	-
785d2	1370	-	-	-	+	+	-	-
951b2	510	-	-	-	-	+	+	-
826f8	1470	-	-	-	-	+	+	+
933c4	1680	-	-	-	-	-	-	+

[a] The YACs in bold are the YACs from which we isolated cDNA (used in subtraction). The other YACs are overlapping YACs within the SRO.

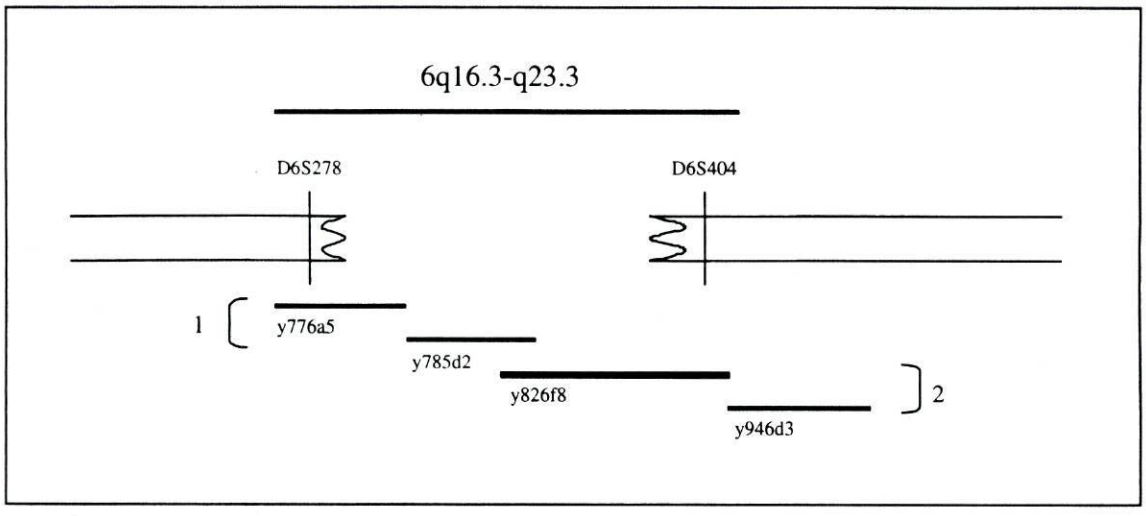


Figure 14. Deleted region in gastric carcinoma (6q16.3-q23), flanked by microsatellite markers D6S278 and D6S404. 1 – Subtraction of YACs 776a5 and 785d2. 2 – Subtraction of YACs 826f8 and 946d3.

Efficiency of subtraction was tested by PCR, using primers specific for the yeast housekeeping gene *ZWF1*. A PCR product with the expected molecular weight was obtained in the unsubtracted cDNA populations and not in the subtracted ones (Figure 15), showing that cDNAs common to both populations were depleted.

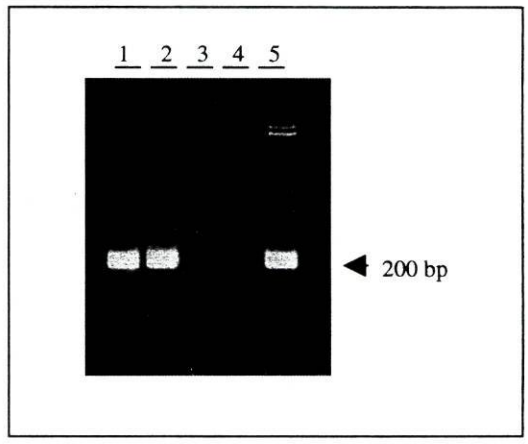


Figure 15. Results from the amplification of *ZWF1* gene on the subtraction of YACs 776a5 and 785d2. Lanes 1 and 2, unsubtracted cDNA from y776a5 (tester) and y785d2 (tester), respectively. Lanes 3 and 4, Subtracted cDNA from y776a5 (tester) and y785d2 (tester), respectively. Lane 5 genomic DNA from y785d2 (control of PCR).

As described in the Material and methods section, libraries of cloned cDNAs were obtained from each subtraction. In the first subtraction (y776a5/y785d2) two libraries, corresponding to the forward and reverse subtractions, were obtained. In each library 192 clones were selected. In the second subtraction (y826f8/y946d3) one library was obtained, corresponding to the forward subtraction. In this library 384 clones were selected. Every clone was checked for the presence and size of the insert by PCR and agarose gel analysis. An example of the variety of insert sizes is depicted in Figure 16.

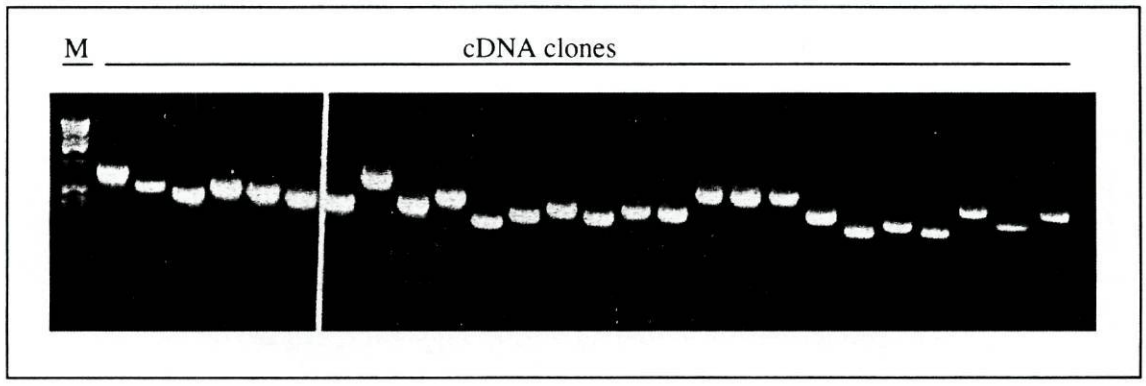


Figure 16. Amplification of cDNA clones with vector primers M13. Clone sizes vary between 100 and 800 bp. M, marker (1 kb ladder).

In the first subtraction, 30 positive clones (14 from the forward subtraction and 16 from the reverse subtraction) were selected by differential screening (hybridization of either the forward or reverse subtracted uncloned cDNA with the forward and reverse cDNA libraries), and in the second one 15 positive clones (from the forward subtraction) were selected. An example of a positive clone selection is shown in Figure 17.

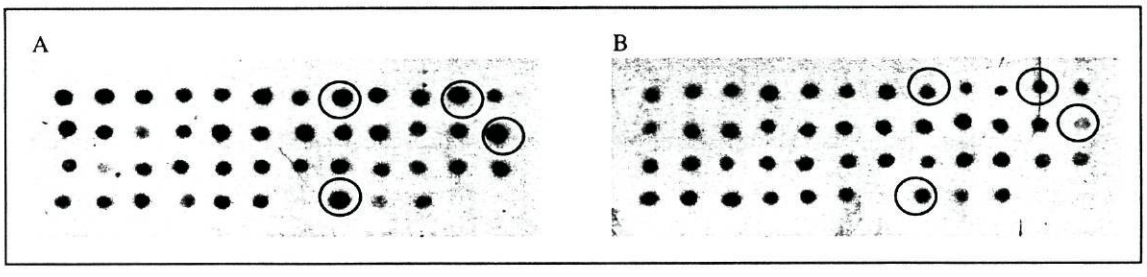


Figure 17. Example of a differential screening hybridization result. Hybridization of the cDNA library of clones specific from yY785d2 (reverse experiment of the first subtraction). A – probe - pool of cDNAs subtracted (not cloned) from the reverse experiment of the first subtraction (y785d2 as tester); B – probe - pool of cDNAs subtracted (not cloned) from the forward experiment of the first subtraction (y776a5 as tester). Examples of some positive clones specific for y785d2 (clones 8, 11, 24, 44) are indicated with a circle.

All 30 positive clones from the forward (n=14) and reverse (n=16) subtractions from the first subtraction, as well as all 15 positive clones from the forward reaction of the second subtraction, were further analyzed.

Results from these analyses are summarized in tables 3, 4, 5, 6, 7 and 8.

From the 14 clones selected in the forward procedure, 7 corresponded to different cDNAs (Table 4). From these 7, 4 hybridized with the human DNA and with the DNA from the tester YAC (y776a5) and 3 hybridized with DNA from all YACs and not with the human DNA. When hybridized with the cDNA library, 3 of the 4 human clones, were present in the library in several copies (Table 4) and only one did not hybridize to other clones from the cDNA library. By sequence analysis we could confirm the 6q origin of all 4 human cDNA clones (Table 5).

Table 4. Southern analysis data of selected clones from the forward experiment of the first subtraction (tester y776a5)

Clones	Size (bp)	HP	Size (kb)	y776a5	y785d2	y798g12	y911f5	y915e4	Similar clones
5	364	+	5.0	+	-	-	-	-	n = 5
26	-	-	-	+	+	+	+	+	-
52	-	-	-	+	+	+	+	+	-
63	-	-	-	+	+	+	+	+	-
91	584	+	12.0	+	-	+	-	-	n = 1
144	282	+	7.0	+	-	-	-	-	n = 0
180	480	+	8.5	+	-	+	-	-	n = 6

Table 5. Homology data of the human clones from the forward experiment of the first subtraction (tester y776a5).

Clones	Homology	% Identity
5	Clone 12803 on 6q21-22	100%
91	Clone RP1-262C15 on 6q16.1-q21	93%
144	Clone RP3-429G5 on 6q21	100%
180	Clone 354J5 on 6q21-22	98%

From the 16 clones selected in the reverse procedure, 14 corresponded to different cDNAs (Table 6). From these 14, 8 hybridized with the human DNA and with the DNA from the tester YAC (y785d2) and 6 hybridized with DNA from all YACs and not with the human DNA. When hybridized with the cDNA library, three of the 8 human clones were present in the library in several copies (Table 6) and 5 did not hybridize to any other clone from the cDNA library. By sequence analysis we could confirm the 6q origin of 4 out of 8 human cDNA clones (Table 7).

Table 6. Southern analysis data of selected clones from the reverse experiment of the first subtraction (tester y785d2).

Clones	Size (bp)	HP	Size (kb)	y776a5	y785d2	y798g12	y911f5	y915e4	Similar clones
6	642	+	3.5	-	+	-	-	-	n = 1
8	422	+	4.0	-	+	-	+	-	n = 0
11	-	-	-	+	+	+	+	+	-
17	562	+	9.0	-	+	-	+	-	n = 0
21	-	-	-	+	+	+	+	+	-
24	810	+	5.5	-	+	-	+	-	n = 2
44	164	+	5.5	-	+	-	+	-	n = 0
68	-	-	-	+	+	+	+	+	-
77	583	+	6.0	-	+	+	-	-	n = 0
78	-	-	-	+	+	+	+	+	-
119	-	-	-	+	+	+	+	+	-
132	584	+	10.0	-	+	-	-	-	n = 0
144	389	+	7.0	-	+	-	+	-	n = 1
146	-	-	-	+	+	+	+	+	-

Table 7. Homology data of the human clones from the reverse experiment of the first subtraction (tester y785d2)

Clones	Homology	% Identity
6	Unknown	-
8	Unknown	-
17	Clone 261K5 on 6q2	99%
24	Repeat sequences in several chromosomes	95%
44	Unknown	-
77	Chromosome 6 TaqI fragment (SC6pA4G2)	95%
132	Clone RP5-919F19 on 6q16.3-22.1	98%
144	Clone CTA-331P3 on 6q21-22.1	96%

From the 15 clones selected in the forward procedure of the second subtraction, 3 corresponded to different cDNAs (Table 8). When hybridized with the cDNA library, all 3 clones were present in the library in several copies (Table 8). By sequence analysis we could confirm the 6q21 origin of all these cDNAs (Table 9). Clones 17 and 178 showed homology to the same PAC clone, although they did not hybridize with each other when hybridized to the all cDNA library.

Table 8. Southern analysis data of selected clones from the forward experiment of the second subtraction (tester y826f8).

Clones	Size (bp)	HP	Size (kb)	y826f8	y946d3	y785d2	y951b2	y933c4	Similar clones
17	336	+	2.5/ 12.0	+	-	-	-	+	n = 16
28	254	+	2.5	+	-	-	+	-	n = 4
178	435	+	12.0	+	-	-	-	+	n = 4

Table 9. Homology data of the human clones from the forward experiment of the second subtraction (tester y826f8).

Clones	Homology	% Identity
17	PAC 66H14 from 6q21-22	98%
28	PAC 487J7 from 6q21-22.1	97%
178	PAC 66H14 from 6q21-22	96%

Figure 18 shows the physical location of the several clones within the target YACs and overlapping ones.

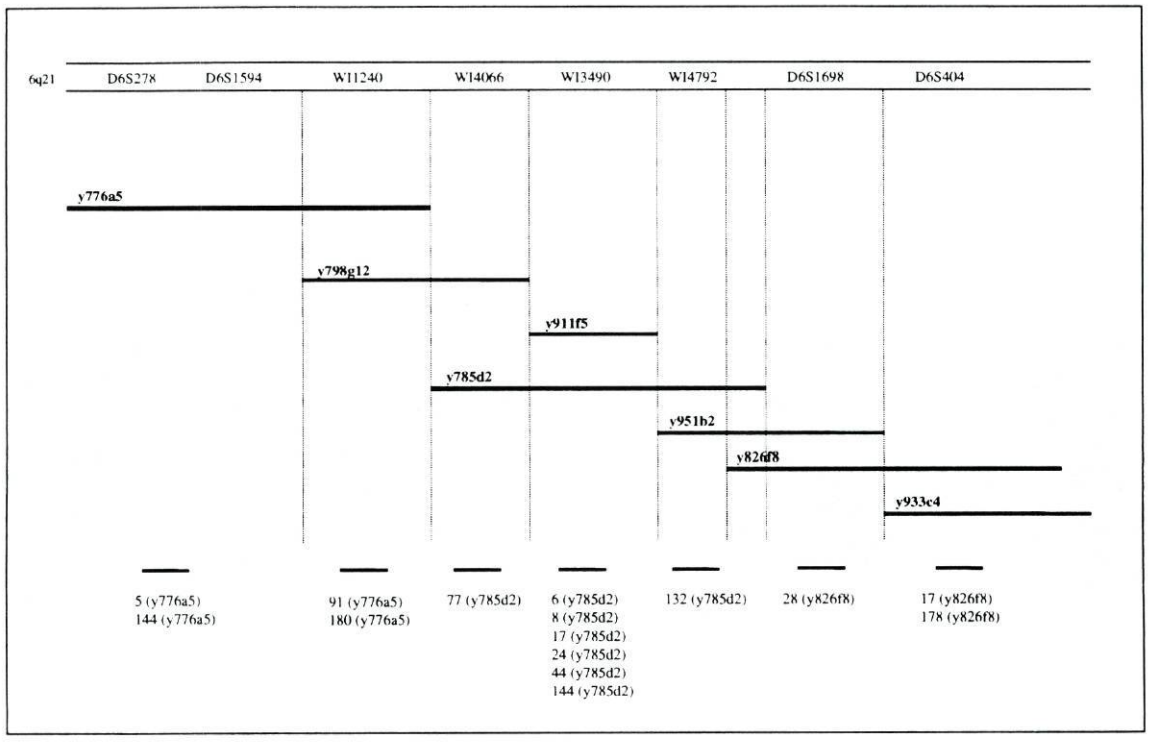


Figure 18. Physical mapping of the clones within the YAC contig that covers the 2-cM SRO.

The human clones were analyzed by Northern analysis by hybridization to multiple tissue Northern blots. From the 14 clones that were analyzed, clone 5 (y776a5) and clones 17, 132 and 144 (y785d2), indeed showed expression in normal tissues of different embryological origin. An example is shown in Figure 19.

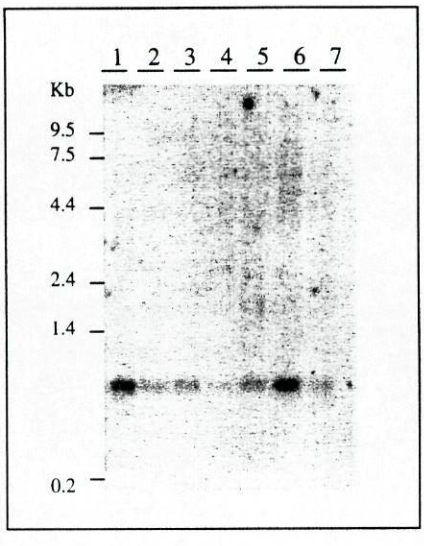


Figure 19. Northern results of clone 144 (y785d2) when hybridized to a multiple-tissue Northern blot. Lane 1, stomach; lane 2, thyroid; lane 3, spinal cord; lane 4, lymph node; lane 5, trachea; lane 6, adrenal gland; lane 7, bone marrow.

DISCUSSION

Gastric carcinoma development is, as in other human neoplasias, a multi-step process in which several genes are altered. Some genes are activated while others may be inactivated, leading to an unbalanced proliferation of the cells and ultimately to the establishment of the tumor. Some knowledge has already been achieved regarding the role of a number of genes in gastric carcinogenesis, like the role of *E-cadherin* in the development of the diffuse histotype (Becker *et al*, 1994; Guilford *et al*, 1998; Machado *et al*, 1999). Still, many questions remain to be answered in order to understand the genetic pathway of gastric carcinogenesis.

The main scope of this thesis was to investigate the role of chromosome 6 in the development of gastric carcinoma, as it is one of the most frequently altered chromosomes in gastric cancer (Ochi *et al*, 1986; Seruca *et al*, 1993; Barletta *et al*, 1993; Panani *et al*, 1995; Seruca *et al*, 1995a; Schneider *et al*, 1995; Gleeson *et al*, 1997).

By loss of heterozygosity (LOH) studies, deletions of the long arm of chromosome 6 were detected in 29-53% of informative cases. They occur in all histologic types of gastric cancer (Seruca *et al*, 1995a; Schneider *et al*, 1995; Gleeson *et al*, 1997) and in early as well as in advanced tumors (Seruca *et al*, 1995a). Our group started the analysis of chromosome 6 in gastric cancer in 1992, and defined two smallest regions of overlap (SRO) of heterozygous deletions (Queimado *et al*, 1995), using restriction fragment length polymorphism (RFLP), variable number of tandem repeats (VNTR) and microsatellite markers. One SRO of approximately 15 cM lies interstitial, at 6q16.3-q23.1, and is lost in 50 % of informative cases. The other SRO spans more than 30 cM, lies distal to 6q23-q24, at 6q27, and is lost in 37% of informative cases. These results suggest the presence of tumor suppressor genes (TSGs) in these regions, putatively involved in gastric cancer development.

1. Delimitation of the SRO at 6q in gastric carcinomas

The first step in a positional cloning approach, is to delimit the region of interest, which is the possible location of the putative gene, to a segment small enough to initiate a gene search. Such a region should not be larger than a few Mb, otherwise it is unsuitable to start the search. As both SROs defined by Queimado *et al* (1995) were still too large, we had to further delimit them.

We focused on the interstitial SRO as it was deleted more frequently in gastric cancer than the distal SRO. The interstitial SRO as defined by Queimado *et al* (1995), is flanked by *loci* D6S268 and *ARG1* and contains *locus* D6S32. The marker for D6S32 is an RFLP marker that was assigned to 6q22.3 by FISH (Rao *et al*, 1993). The heterozygosity frequency of this marker is low (38%) compared to microsatellite markers, which often have heterozygosity frequencies of well over 75%. Thus, we wanted to assemble a panel of polymorphic microsatellite markers (Gyapay *et al*, 1994), that would map within the region of the interstitial SRO. For that we first tried to determine the position of D6S32 relative to the latest versions of the physical maps that contain the microsatellite markers. In the available databases (cedar database – http://cedar.genetics.soton.ac.uk/pub/chrom6/map.html; genome database – http://gdbwww.gdb.org/) D6S32 was recently listed to map at 6q23.3, instead of 6q22.3. To be able to more accurately compare the "old" D6S32 data with the more recent data on microsatellite analyses, we thought it would be worthwhile to map this marker. None of the YACs that we collected in the course of our project hybridized with D6S32. These analyses included YACs that map within the region where D6S32 was recently assigned, as well as YACs within the SRO defined by Queimado *et al* (1995). Although the location of D6S32 was still unknown, we could already rule out its location in this segment of 6q. The screening of PAC libraries, that would have given us the opportunity to continue the mapping with a much larger genomic fragment, was unsuccessful. Finally, when we determined the nucleotide sequence of D6S32 and used it to screen various databases, we identified the BAC clone 2023J2. No mapping data were available for this BAC that was only deposited recently in the GSS (Genome Survey Sequence) database at NCBI (National Center for Biotechnology Information). However, FISH analysis using this BAC in combination with YAC clones that we now have available, will tell us the true position of D6S32 within the genetic/physical map of 6q. Because of the uncertainty of the location of D6S32 we focused instead on the flanking markers of the SRO, and therefore selected 19 markers from the databases, that covered this segment of chromosome 6.

The analysis of 60 primary gastric tumors with the microsatellite markers spanning the region 6q16.3-q23.3, showed that 50 cases were informative for at least one *locus*, and 18 of these (36%) presented allelic imbalance (AI). By comparing these 18 we were able to define a SRO of 2 cM (approximately 3 Mb), flanked by markers D6S278 and

D6S404. The AI can be interpreted either as gain or as loss of alleles. In this study it was interpreted as allelic loss rather than gain of alleles based on two arguments. First, because four of the cases analyzed were previously studied, either by RFLP markers or by cytogenetics, and showed clear loss of 6q material (Seruca *et al*, 1993; Seruca *et al*, 1995a; Queimado *et al*, 1995). These analyses are less affected by the admixture of normal and tumor tissues. Moreover, 14 cases, that contributed to the mapping of the 2 cM SRO, had regions of retention along 6q. This rules out an AI due to a duplication of the complete q-arm of chromosome 6 (duplications are most often due to the excess of whole chromosomes or whole chromosome arms). Thus, these data are in support of a region with a high frequency of loss of heterozygosity in gastric cancer.

We compared the relative frequencies of AI for 6q (markers within the SRO) with the frequency of AI at two other hotspots of heterozygous deletions in gastric cancer – 3p21.3 and 17p12-p13. The frequencies of AI detected were 15%, 36% and 38% for 3p, 6q and 17p, respectively. Since the frequencies of AI detected at 3p and 17p are low compared to what has been previously published (Schneider *et al*, 1995; Seruca *et al*, 1995a; Gleeson *et al*, 1997), we can assume that the 36% of AI detected at 6q is probably underestimated. This may be due to the number of tumor specimens in which the amount of stromal cells precludes detection of AI. Thus, we can expect that AI at 6q is much more frequent supporting an important role in gastric cancer development.

Like Queimado *et al* (1995) we also detected AI both in early and advanced tumors, indicating that loss of sequences from 6q is an early event in gastric carcinogenesis (Queimado *et al*, 1995; Seruca *et al*, 1995a). 6q deletions were found in 10/30 (33%) intestinal tumors, 4/15 (27%) atypical tumors and 4/15 (27%) diffuse tumors, showing that 6q alterations play a role in the development and/or progression of the neoplasia, regardless the histological type.

Deletions at 6q are also described for melanomas (Trent *et al*, 1989), mesotheliomas (Taguchi *et al*, 1993), lymphomas (Gaidano *et al*, 1992), leukemias (Menasce *et al*, 1994b), breast and ovarian carcinomas (Orphanos *et al*, 1995a, 1995b), salivary gland carcinomas (Jin *et al*, 1994) and pancreatic carcinomas (Griffin *et al*, 1994). In Figure 20 SROs defined at 6q21 in different studies are compared. Some SROs overlap, at least partly with the SRO defined in this study. The fact that the same region is affected in tumors of different types of tissue (lymphoid malignancies, mesotheliomas and carcinomas) can indicate that the genes putatively involved are not tissue-specific but may play a more general role in cancer development.

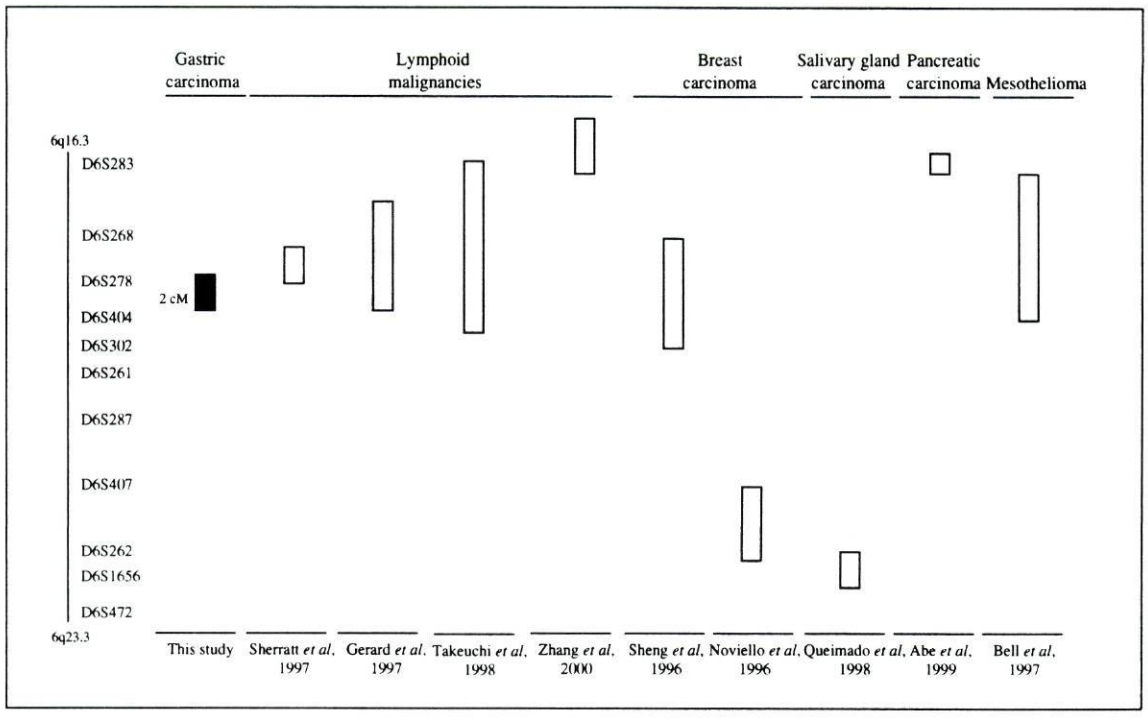


Figure 20. Diagram of defined SROs at 6q16.3-q23.3 in different studies.

Several candidate tumor suppressor genes mapped at 6q16.3-q23 region may actually map within the SRO that we defined. The *AIM1* gene (Ray *et al*, 1996) has been related to the development of malignant melanomas, where it is frequently deleted. We tried to check the position of *AIM1* relatively to our SRO. However, none of the YACs mapping within our 2-cM SRO, could be amplified by PCR using primers designed for *AIM1*. This indicates that *AIM1* does not map in the SRO defined by us. Moreover, according to the genetic map of Science 98 (http.// www. ncbi. nlm. nih. gov/ genemap 98) *AIM1* (6q21) maps centromeric to D6S268, which lies 2 cM proximal to D6S278 that flanks the SRO proximally, supporting the exclusion of this gene as the putative gene deleted in gastric carcinoma.

2. Analysis of 6q alterations in xenografts and cell lines

Our group has xenografted six primary gastric tumors into nude mice. From these xenografts, three cell lines were established. This type of material is very valuable for further studies for several reasons: (1) it does no longer have admixture of normal tissue; (2) it is an unlimited source of material; and (3) it may give information about the progression of the tumor, since a positive selection for clones with a greater growth potential can occur. We decided to analyze the chromosome 6 alterations in these xenografts and cell lines, in order to see if they presented the alterations that we frequently detected in the primary tumors.

By microsatellite analysis we observed that three cases (GP199, GP226 and GP233) either did not show any imbalance (in case of GP226) or presented microsatellite instability (cases GP199 and GP233). The cases presenting MSI^+ phenotype will be helpful in the study of the MIN pathway of tumor development. Despite showing retention for almost all *loci* analyzed, deletions at the defined SRO can not be ruled out in case 226, since the markers within the region were not informative in this case (Figure 10). The other cases (GP195, GP202 and GP 220), when analyzed by microsatellite analysis (xenografts and cell lines) and by FISH (cell lines), showed imbalances for the long arm of chromosome 6. FISH results (Figure 12) showed that a large part of 6q appeared *in triplo* in all cell lines, presumably as a result of a partial chromosome duplication. For GP195 the duplication included the centromere. Two cell lines and the corresponding xenografts (GP195 and GP202) appeared to have lost one of the parental regions 6q27-qter, which is seen as a complete LOH of this segment, by microsatellite analysis. None of these alterations were detected in the respective primary tumors. Only in one tumor (case 220), where microdissected material was available, allelic imbalance was detected in the primary tumor (Figure 11).

The seeming discrepancy between the findings in the primary tumors on one hand, and those in the xenografts and cell lines on the other hand, may be explained by a masking of the allelic imbalance in the primary tumors as a consequence of a large admixture of normal cells. This may in particular occur in diffuse type carcinomas (such as the primary tumor of case 220), where neoplastic cells are isolated and dispersed in stromal tissue. When tumor sections of this case were carefully microdissected, the allelic imbalance present in the xenograft could also be detected in the primary tumor. Another explanation may be that primary tumors are heterogeneous with respect to their

genomic alterations, and that clonal selection has occurred during the growth of the xenograft. Case 202 may be an example of this, since the primary tumor was classified as atypical with a component of diffuse cells, whereas in the matching xenograft only diffuse type cells were observed. Microsatellite analysis cannot by itself, distinguish between these two possibilities, which may in any case be both true.

The FISH results clearly show that the AI detected in this analysis represent a gain of 6q material, rather than allelic loss as described above for the primary tumors. These results do emphasize that care must be taken when interpreting microsatellite analysis results, since an imbalance ratio lower than 2, like the ones we obtained (Figure 11) does not necessarily mean LOH for that region. As clearly shown by our FISH results, allele ratios lower than 2 may also result from duplication of the analyzed *loci*.

Nonetheless, we still regard the AI detected in the primary tumors to be a true loss as the situation is noticeably different. In the primary tumors, we often detected AI for a small region of 6q that was flanked by regions of retention. Such a pattern is highly suggestive of true loss rather than a duplication of only a small segment of the q-arm. This interpretation was further supported by the fact that in some of the samples LOH with RFLP markers from the same region had previously been shown (Queimado *et al*, 1995).

The overrepresentation of the long arm of chromosome 6 observed in the three cell lines may indicate that during the *in vivo* and *in vitro* culture, clones with this region in triplicate, undetectable in the primary tumors, had a selective advantage. Though the number of cell lines is limited, this consistent overrepresentation of the 6q16.3-q23.3 region, particularly in the near-diploid cell line GP220, suggests the presence of (a) proliferative gene(s). On the other hand, two of our cell lines, GP202 and GP195 (Gärtner *et al*, 1996 and Figure 12) have a widespread overrepresentation of chromosomes. Thus, at least for these two cell lines it cannot be ruled out that the observed gain of 6q material is part of a general increase of ploidy of the cells rather than an isolated event related to tumor development or progression.

In addition to the duplication of 6q material from one of the parental chromosomes, cases 195 and 202 showed complete loss of one of the parental alleles at the terminal part of the long arm of chromosome 6. The terminal 6q region, already pinpointed by Queimado *et al.* (1995) as being frequently lost in gastric carcinoma, may therefore harbor a tumor suppressor gene. This loss of heterozygosity may have preceded the duplication event. That is, a TSG would be lost in earlier stages of tumor development

followed by duplication of a proliferative gene(s) that would have a role in progression, as schematically represented in Figure 21, if we take into account that 6q duplication is not a random event due to gross aneuploidy.

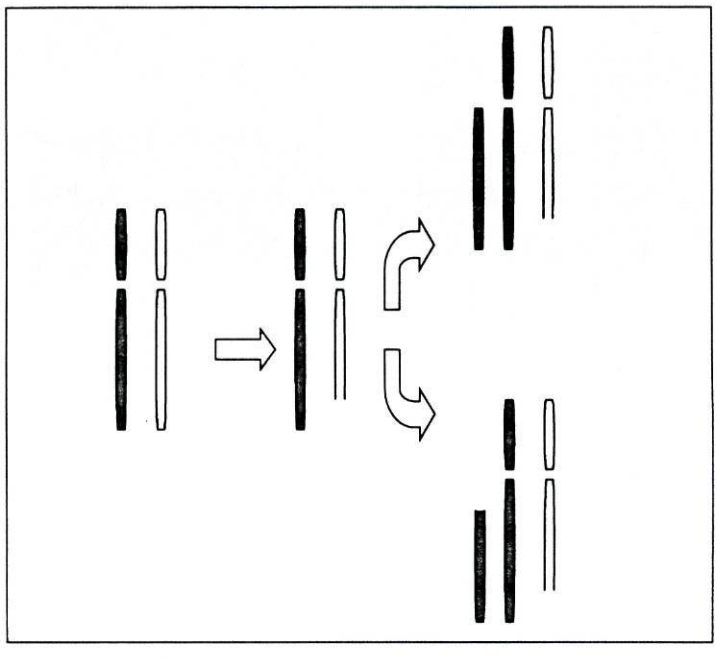


Figure 21. Schematic representation of hypothetical tumor progression regarding chromosome 6 alterations in cases GP195 and GP202.

The availability of these two gastric cancer-derived cell lines with aneuploidy of the interstitial region and a terminal deletion of 6q may be useful for further –functional- studies aiming at the identification of the putative tumor suppressor gene(s) in the distal region of 6q.

There are already some genes identified at 6q27 that were pinpointed as putative tumor suppressor genes, like *AF6* (Prasad *et al*, 1993), *M6P/IGF IIR* (Hankins *et al*, 1996; De Souza *et al*, 1995b) and *SEN6* (Banga *et al*, 1997), mentioned previously in this thesis. *AF6* gene was described in leukemias, as being involved in a translocation (Prasad *et al*, 1993). *M6P/IGF IIR* encodes for a growth factor receptor that has a role in negative cell growth control and was implicated in the development of breast (Hankins *et al*, 1996) and hepatocellular (De Souza *et al*, 1995b) carcinomas. *SEN6*, a gene for

the cellular senescence in human cells, may be responsible for the immortalization of several types of tumors that present deletion of 6q27 (Banga *et al*, 1997). The putative role of these genes in gastric cancer development needs to be analyzed. The two xenografts and the matching cell lines cell lines that present deletion of this region (GP195 and GP202) may form a good starting point for these analyses.

3. Construction of the transcription map of the 2-cM SRO

In order to determine which genes map in the 2 cM region, defined in this study, we decided to construct a transcription map for that region. Genes were isolated by a modification of a subtraction protocol, in a way that, instead of analyzing the whole genome using target tissues or templates (normal *versus* tumor), only the 2 cM region on 6q could be analyzed. Knowing that human genes present in YACs are transcribed in the yeast strain (Still *et al*, 1997), we decided to subtract yeast strains containing different YACs. With this strategy we expected to obtain at the end of the subtraction a pool of cDNAs enriched for the genes encoded by the human inserts of the YACs.

First, we selected contiguous and non-overlapping YACs that were mapping in the 2-cM SRO. Three YACs covered the region – y776a5, y785d2 and y826f8 (Figure 14).

Secondly, we performed suppression subtractive hybridization (SSH) using cDNA of these YACs as tester and driver cDNAs. We chose SSH because this method is a simple and quick procedure and has advantages compared to other subtraction methods: (1) it normalizes (equalizes) sequence abundance among the target cDNA population, which allows the detection of rare transcripts; (2) just needs one round of subtraction; and (3) does not need an extra step for physical separation of the unhybridized cDNAs (differentially expressed transcripts) due to the suppression effect during PCR. At the end of the procedure an enrichment of 1000-fold or more for the differentially expressed cDNAs can be achieved (Diatchenko *et al*, 1996).

Diatchenko *et al* (1996) used this method to subtract cDNAs synthesized from human testis poly(A)$^+$ RNA against a mixture of cDNA derived from poly(A)$^+$ RNA of 10 different human tissues, and achieved a high level of enrichment of testis-specific cDNAs. They used the subtracted cDNA mixture to screen a cosmid library constructed from flow-sorted human Y chromosomes in order to identify functional sequences expressed in testis and thus identified 5 chromosome Y-specific genes expressed in testis. The application of this method also led to the identification of 27 genes differentially expressed in an estrogen receptor-positive breast carcinoma cell line when

compared with an estrogen receptor negative breast carcinoma cell line (Kuang *et al*, 1998).

After having done two subtractions (each with forward and reverse experiments), we obtained differentially expressed cDNAs from y776a5 (first subtraction, forward experiment), y785d2 (first subtraction, reverse experiment) and y826f8 (second subtraction, forward experiment). These cDNAs were cloned and further analyzed. Differentially expressed cDNAs from y946d3 (second subtraction, reverse experiment) were not included in the analysis because this YAC maps outside the region of interest.

The efficiency of the subtractions was verified by PCR analysis of the yeast housekeeping gene *ZWF1* (homologous to the human glucose-6-phosphate dehydrogenase gene) on the subtracted and unsubtracted cDNAs. No PCR product was detected when the subtracted cDNA was used as a template, suggesting that indeed a depletion of commonly expressed sequences was achieved at the end of the two subtractions.

After selection and confirmation of YAC specificity of the clones, we ended up with 45 clones. Some clones were present only once in the cDNA libraries but others were present more than once. From the 45 selected clones, 24 were independent and from these 15 were of human origin as they hybridized with human DNA and the tester YAC. Nine of the 24 selected clones hybridized to all yeast DNA samples present on the membrane, and not to human DNA. These clones presumably represent yeast cDNAs. This conclusion was confirmed by sequence analysis of some of these clones (data not shown), that indicated a high degree of homology with yeast sequences. The detection of yeast cDNAs may be caused by the differential expression of some yeast genes between the two cultures or may be intrinsic to the method that only guarantees an enrichment in differential expressed genes.

We compared the sequence of the human clones with the database (BLAST search) and could confirm the 6q origin of 11 clones. These clones showed homology to uncharacterized clones deposited in the database. These clones might correspond to unknown cDNAs that await identification.

Still within the group of 15 clones, 3 did not show homology to other known sequences (clones 6, 8 and 44 from y785d2). These should be considered human sequences since they only hybridized to the tester YAC and human DNA. They probably consist of sequences that are not yet deposited in the database.

The last clone from the 15 human clones showed homology with a set of different chromosomes. This clone (clone 24 from y785d2) showed high homology with LTR7-HERVH sequences, that are present in multiple copies throughout the genome.

In order to analyze how many of these selected clones indeed represented human genes we performed Northern analysis.

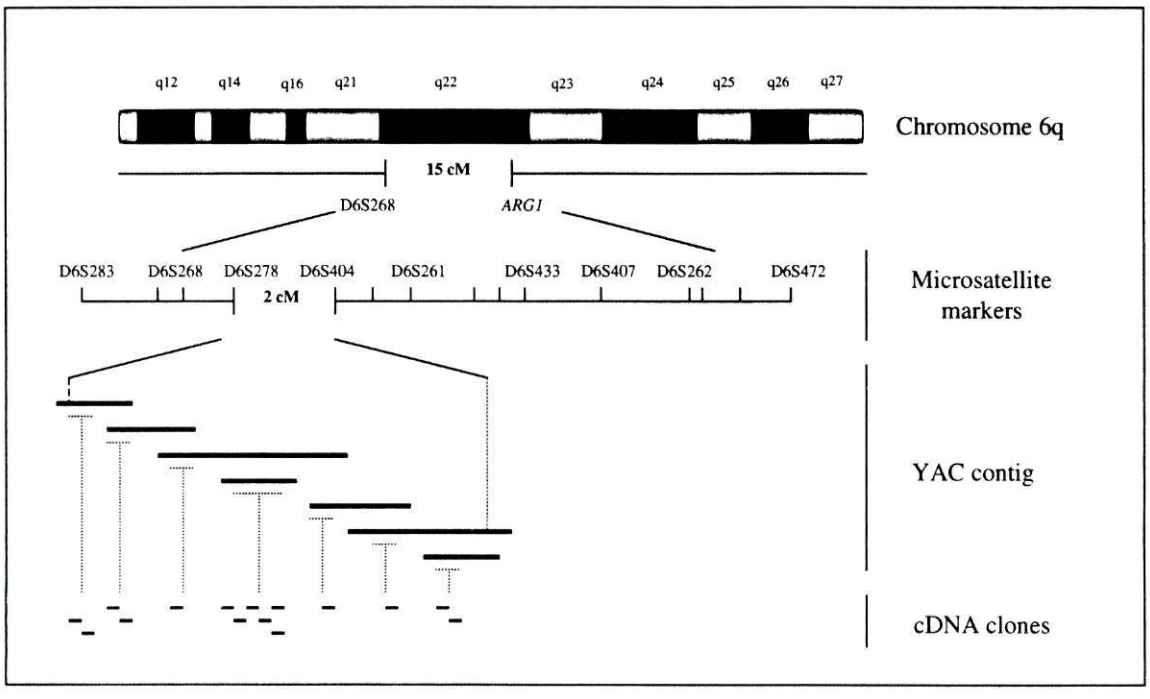
Four out of the 14 clones that were subjected to a Northern analysis showed expression in different tissues. Clone 24 (y785d2) was not subjected to Northern analysis, as we would obtain a smear due to its repetitive content. Our failure to detect expression for 10 clones can be caused by their overall low expression in human tissues in combination with the small size of the clones (probes). Alternatively, some of the clones may consist of intronic sequences that were not spliced out during the mRNA maturation process, for it is known that splicing of foreign transcripts in yeast is not efficient (Langford *et al*, 1983). Human and mouse sequences lack some of the consensus sequences that the splicing machinery in yeast needs to properly excise the introns (Langford and Gallwitz, 1983; Trachtulec and Forejt, 1999).

All the 4 clones with expression in normal tissues showed expression in stomach, albeit only weak for clone 5 from y776a5. It is crucial to evaluate the expression of these 4 clones in gastric tumor tissue and in gastric tumor cell lines. If an altered expression in tumorigenic tissues is observed in these cDNAs, it would be of interest to obtain their full-length sequences. This would allow us to predict a possible function of the transcripts and later be able to perform mutational and functional analyses.

Summing up, with the application of the SSH method using YACs mapping at the 2-cM SRO at 6q we were able to isolate 15 human transcripts from that region, 4 of which showing expression in the stomach. These cDNAs represent good candidates for the putative genes involved in gastric carcinogenesis.

4. Conclusions and future perspectives

Altogether, in a positional cloning approach, we were able to delimit the interstitial SRO to a segment of 2 cM (approximately 3 Mb). We isolated 15 putative ESTs; for four of them we could already demonstrate expression in stomach. The shortcut of the method of suppression subtractive hybridization (SSH) applied in the isolation of cDNAs, using YACs from a well defined region, showed to be successful. A summary of the strategy used in this thesis is depicted in the scheme below.



The study of cell lines derived from xenografted gastric tumors reinforced the importance of the deletions of the terminal region of 6q in the development of gastric carcinoma.

As concerns future studies on the involvement of long arm of chromosome 6 several analyses should be carried out:

- Detailed expression studies of the cDNAs that map in the 2-cM SRO, focusing on their expression in normal mucosa of the stomach and in gastric tumor tissues (primary tumors, xenografts/cell lines), in order to assess their role in gastric cancer development.
- Isolation of the full-length sequences of the cDNAs with altered expression in gastric tumors, in order to elucidate the gene structure and facilitate mutational and functional analyses.
- Expression analysis of genes reported by others to map at 6q and to be putatively involved in cancer development in the xenografts and the gastric cancer derived cell lines.

- Characterization of the distal SRO described previously since, as supported in this study, this region might harbor (a) putative tumor suppressor gene(s). This characterization may be done in two ways: (a) by detailed deletion mapping followed by isolation of cDNAs from the delimited region, as it has been done on the interstitial SRO in this study; (b) by arrayCGH analysis, where arrays of DNA from BACs covering the distal region are spotted and hybridized with tumor DNA in order to select the region(s) in which genes involved are located.
- It can be foreseen that expression studies of candidate gastric cancer suppressor genes have to extend to primary tumors. The availability of a panel of gastric cancer specimens from which good quality RNA can be obtained, will be of crucial importance.

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SUMÁRIO E CONCLUSÕES

O cromossoma 6 é um dos cromossomas mais frequentemente envolvido em alterações estruturais em tumores gástricos. De todas as anomalias descritas deste cromossoma, a delecção parcial do braço longo representa é a alteração mais vezes detectada, tanto por análise citogenética como por estudos de perda de heterozigotia (LOH).
A perda de heterozigotia em 6q ocorre em todos os tipos histológicos de carcinoma gástrico, incluindo tumores precoces.

O objectivo "major" deste estudo foi o isolamento de genes localizados em 6q, potencialmente envolvidos no desenvolvimento e progressão de tumores gástricos.

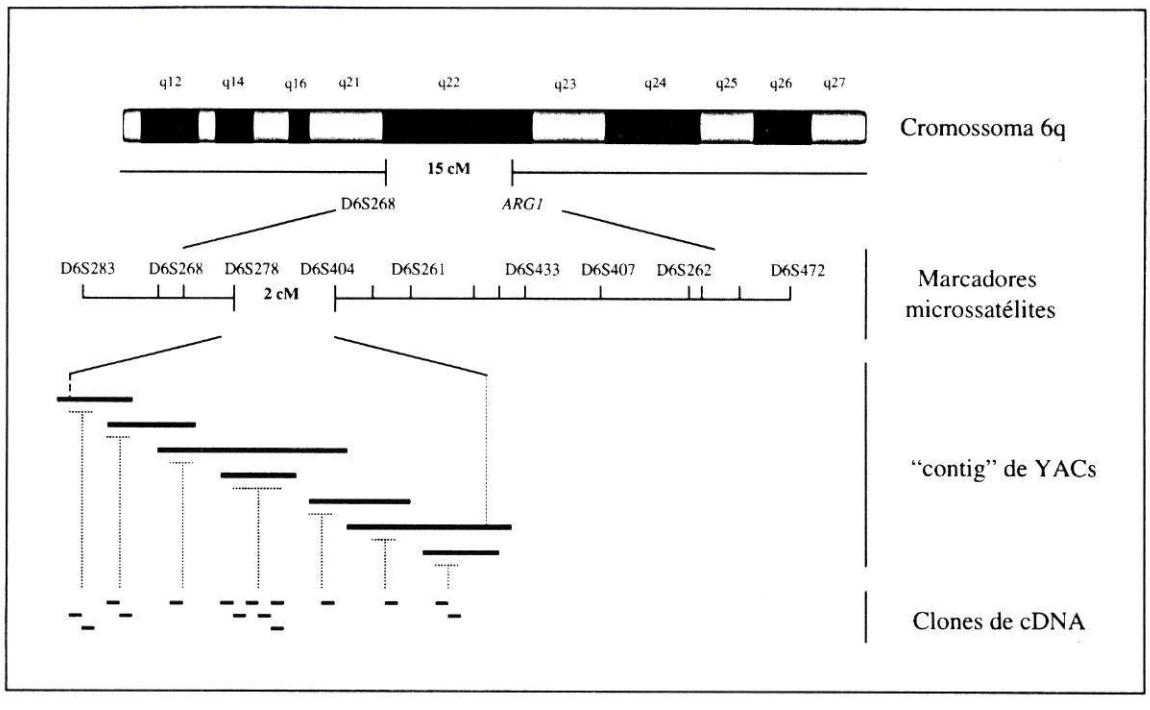
Em estudos anteriormente realizados pelo nosso grupo, foram definidas duas regiões de delecção mínima – uma intersticial em 6q16.3-q23.1 (15 cM) e uma distal em 6q27 (> 30 cM). No entanto, como a extensão destas duas regiões não permitia ainda o início de pesquisa de genes, o nosso primeiro objectivo foi delimitar a região intersticial. Para isso, foram seleccionados novos marcadores localizados na região (marcadores microssatélites), que viriam a ser aplicados na análise de um painel alargado de tumores primários. **Do mapeamento detalhado da região foi possível restringir a região intersticial de 15 cM para uma nova região de apenas 2 cM (aproximadamente 3 Mb)** (Artigo I).

No sentido de ultrapassar os problemas de análise e interpretação decorrentes da contaminação estromal em tumores primários, procedemos ao estudo de tumores xenografados em ratinhos atímicos e linhas celulares derivadas dos xenografos, estabelecidos no nosso Instituto. Este estudo, envolvendo análise de microssatélites e FISH, permitia verificar se as linhas celulares em causa apresentavam as mesmas alterações em 6q detectadas nos tumores primários, o que, a verificar-se, permitiria utilizar este material como para estudos futuros, nomeadamente estudos funcionais.

Verificámos que, tal como nos tumores primários, duas linhas celulares apresentavam uma delecção distal (6q27). **Estes resultados apoiam a existência de um ou mais genes supressores tumorais na região distal do braço longo do cromossoma 6, possivelmente envolvido(s) no desenvolvimento do carcinoma gástrico** (Artigo II).

Com o objectivo de isolar genes envolvidos no desenvolvimento do carcinoma gástrico, iniciámos a construção de um mapa de transcrição da região de 2 cM. Para tal, foi utilizada **uma técnica de subtracção (*suppression subtractive hybridization* –SSH) na análise de uma região de aproximadamente 3 Mb, que permitiu o isolamento de cDNAs humanos localizados na região intersticial (6q16.3-q23.3)** (Artigo III). O padrão de expressão dos clones isolados mostrou que 4 clones apresentavam expressão na mucosa gástrica normal. Estes 4 cDNAs constituem possíveis genes candidatos com função importante no desenvolvimento e progressão do carcinoma gástrico. No entanto, será ainda necessário realizar estudos adicionais que permitam confirmar esta hipótese.

Em resumo, a estratégia de clonagem posicional utilizada neste estudo (ilustrada no esquema seguinte) permitiu-nos delimitar a região de 15 cM para uma região de delecção mínima de 2 cM e isolar cDNAs correspondentes a genes expressos na mucosa do estômago e possivelmente envolvidos no desenvolvimento e progressão do carcinoma gástrico.



PAPER I

Substantial Reduction of the Gastric Carcinoma Critical Region at 6q16.3–q23.1

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Deletions of the long arm of chromosome 6 are a common event in gastric carcinomas. In a previous study, deletion mapping of 6q identified two smallest regions of overlap (SROs) of heterozygous deletions: one interstitial, spanning 12–16 cM, bordered by *D6S268* (6q16.3–q21) and *ARG1* (6q22.3–q23.1), and one distal to *IFNGR1* (6q23–q24), spanning more than 30 cM. Loss of heterozygosity (LOH) of the interstitial SRO was detected in 50% of informative tumors. We analyzed 60 primary gastric tumors with 19 highly polymorphic markers from 6q16.3–q23.3 to delimit the interstitial SRO further. Of the 50 tumors that were informative for at least one locus, 18 (36%) showed allelic imbalance (AI). The overlap of these cases allowed us to define an SRO of approximately 3 Mb flanked by *D6S278* and *D6S404*. AI or LOH of this region occurs in all histologic types of gastric carcinoma and in early stages of development, indicating that loss of a gene from this region of 6q is a crucial step in a main route of gastric carcinogenesis. For cases with retention of 6q, alternative routes of gastric carcinogenesis may exist. *Genes Chromosomes Cancer 26:29–34, 1999.*

INTRODUCTION

Gastric carcinoma is one of the most frequently occurring neoplasias in Portugal (Instituto Nacional de Estatística, 1992; Da Motta, 1994). Within Europe, Portugal is the country with the highest incidence and mortality rates for gastric cancer (31.9 and 26.2 per 100,000, respectively; Black et al., 1997). Conventional cytogenetic studies, predominantly carried out on advanced-stage tumors, have indicated the presence of numerous and complex chromosomal alterations in this type of cancer (Seruca et al., 1993, 1995a; Peddanna et al., 1995). Microscopically detectable deletions of the long arm of chromosome 6, consistently involving chromosomal bands 6q21–qter, have been observed in 27%–45% of cases (Ochi et al., 1986; Seruca et al., 1993; Panani et al., 1995). An extensive analysis of loss of heterozygosity (LOH), which focused on loci either harboring known tumor suppressor genes or involved in chromosomal aberrations, indicated a high percentage of allelic losses at 6q, 3p, and 17p, namely 32%, 57%, and 44%, respectively (Seruca et al., 1995a). LOH at 6q is detected in all histologic types of gastric carcinoma (Seruca et al., 1995a; Gleeson et al., 1997) and in both early and advanced tumors (Seruca et al., 1995a).

The frequent loss of genetic material from the long arm of chromosome 6, not only in gastric carcinoma but also in other human neoplasias (Trent et al., 1989; Devilee et al., 1991; Saito et al., 1992; Offit et al., 1993), suggests that the long arm of chromosome 6 harbors one or more tumor suppressor genes. Results from microcell-mediated chromosome transfer studies, using melanoma and breast carcinoma cell lines, support this suggestion (Trent et al., 1990; Negrini et al., 1994).

On chromosome 6, we have previously identified two smallest regions of overlap (SROs) of heterozygous deletions (Queimado et al., 1995). One region, which is heterozygously lost in 37% of informative cases, is defined as being distal to *IFNGR1* (6q23–q24) and spans more than 30 cM. The other region, heterozygously lost in 50% of informative cases, is bordered by *D6S268* (6q16.3–q21) and *ARG1* (6q22.3–q23.1) and spans 12–16 cM. Here we describe a more detailed allelic imbalance (AI) analysis of a series of 60 primary tumors, with 19 highly polymorphic markers, and have further delimited the latter region.

MATERIALS AND METHODS

DNA Samples

Sixty primary gastric carcinomas (30 intestinal carcinomas, 15 diffuse carcinomas, and 15 atypical carcinomas) and corresponding normal tissue samples were obtained from surgical resections

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performed from 1988 to 1994 at the Hospital S. João, Porto, Portugal. The tumors were histologically classified according to Laurén's classification (Laurén, 1965). DNA extraction was performed according to standard procedures (Müllenbach et al., 1989). In cases that appeared to have fewer than 50% neoplastic cells (diffuse-type carcinomas), DNA was isolated from microdissected material.

Microdissection

Five-micrometer histologic sections of diffuse-type tumors were obtained from paraffin blocks and stained with hematoxylin-eosin (H&E) for evaluation of the quantity of tumor cells. Areas rich in tumor cells were selected. Serial sections (10 μm) from the same paraffin blocks were made and, by comparison with the stained H&E section, areas of tumor cells were scratched out and used for DNA extraction. The last section (5 μm) was again H&E-stained as control. Paraffin was extracted from the section with xylene, and DNA was isolated by phenol-chloroform extraction (Jen et al., 1994).

Microsatellite Analysis

Nineteen highly polymorphic microsatellite markers mapping on the long arm of chromosome 6 were selected from the human genetic map of Gyapay et al. (1994) (*D6S283*, *D6S447*, *D6S268*, *D6S278*, *D6S404*, *D6S302*, *D6S261*, *D6S454*, *D6S304*, *D6S287*, *D6S433*, *D6S407*, *D6S262*, *D6S472*), Dib et al. (1996) (*D6S1594*, *D6S1647*, *D6S1698*, *D6S1656*), and from the Cedar database accessible at http://cedar.genetics.soton.ac.uk/pub (*D6S457*). Their order, with the approximate genetic distances as far as they are known, is derived from the Cedar database and is indicated in Figure 1. The most probable cytogenetic interval in which the markers reside is 6q16.3–q23.3. A subset of the carcinomas was also analyzed with the markers *TTA1*, *CA9*, and *CA2* on 3p21.3 (Kok et al., 1999) and with the markers *D17S520* and *D17S513*, flanking *TP53* on 17p12–p13.

Polymerase chain reactions (PCRs) were carried out in 20-μl reaction volumes containing 10-mM Tris-HCl (pH 8.0), 50-mM KCl, 1.5-mM $MgCl_2$, 200 mM of each deoxynucleotide triphosphate except dCTP, 20-mM dCTP, 1 μCi of α-^{32}P dCTP, 0.4 mM of each primer, 0.5 U of Taq DNA polymerase (Pharmacia), and 50–100 ng of genomic DNA. Amplification was for 30 cycles with denaturation at 94°C for 0.5 min, annealing at 53°C (*D6S283*), 55°C (*D6S268*, *D6S404*, *D6S302*, *D6S261*, *D6S454*, *D6S304*, *D6S287*, *D6S433*, *D6S262*), 56°C (*D6S1594*, *D6S1698*), 58°C (*D6S278*, *D6S407*, *D6S1656*), or 60°C (*D6S447*, *D6S1647*, *D6S457*, *D6S472*) for 0.5 min, and extension at 72°C for 1.5 min. The initial denaturation step at 94°C and the final extension step at 72°C lasted for 5 min. Aliquots of the PCR mixture were mixed with formamide loading buffer in a 1:1 ratio and denatured at 94°C for 5 min. Three microliters of this solution were subjected to electrophoresis on denaturing 6% polyacrylamide gels. Gel patterns were visualized by autoradiography.

Allelic imbalance (AI) was established if the intensity ratio of the two alleles in the tumor sample differed markedly from the ratio in the normal DNA. This was evaluated by densitometric analysis on an Ultrascan XL LKB densitometer using Gel Scan XL 2.1 software (Pharmacia LKB). An imbalance factor, calculated as the ratio of allele intensities in the tumor relative to the ratio of allele intensities in the normal tissue ([T1/T2]/[N1/N2]), was interpreted according to Van den Berg et al. (1996). A value higher than 1.2 or below 0.8 was considered to represent AI. A value between 0.95 and 1.05 was considered to represent retention. Values between 1.05 and 1.2, or between 0.8 and 0.95, were considered to represent an ambiguous situation and were excluded from analysis. Because interpretation of data is difficult in cases in which the length of the alleles in normal tissue differs by only one or two dinucleotides, these cases were considered uninformative. Tumor samples displaying microsatellite instability (MIN) were also considered uninformative.

RESULTS

In 10 of the 60 primary gastric carcinomas, we detected MIN for the majority of markers. These carcinomas were excluded from the AI analysis. All of the 50 remaining cases were informative for at least one of the 6q markers. In 18 cases, including 10 intestinal carcinomas, 4 atypical carcinomas, and 4 diffuse carcinomas, we detected AI for at least one locus. Within this group of cases, the highest frequencies of AI were observed at *D6S1647* (seven of eight informative cases; 88%), *D6S1698* (four of five informative cases; 80%), and *D6S454* (seven of eight informative cases; 88%). Figure 1 shows the results obtained for these 18 tumors. Representative autoradiograms are presented in Figure 2. Two cases (149, 161) showed AI at all informative loci, indicating loss of the whole region. In 10 cases (11, 15, 29, 166, 197, 209, 220, 223, 228, 231), AI was observed in only part of the region (proximal or interstitial). The remaining six cases (93, 175, 200,

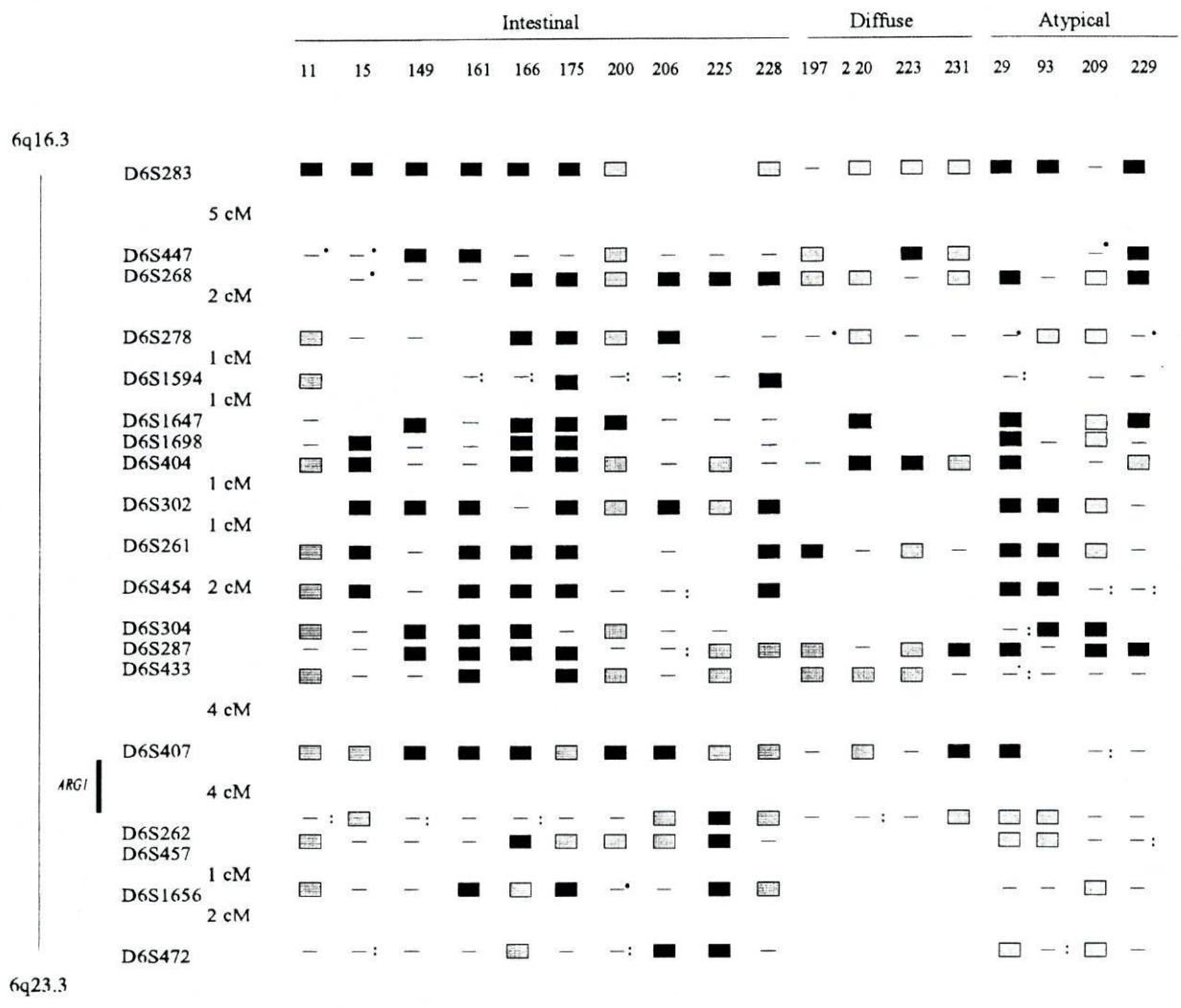

Figure 1. Patterns of allelic imbalance (AI) at 6q16.3–q23.3. ▩, AI; □, retention of heterozygosity; –, not informative (homozygous); –:, not informative (alleles separated only by two base pairs); ., microsatellite instability (MIN); intestinal, tumors of intestinal histologic type; diffuse, tumors of diffuse histologic type; atypical, tumors of atypical histologic type; shadowed region, smallest region of overlap (SRO) of heterozygous deletions. The relative order of the markers was based on the genetic map of the Cedar database.

206, 225, 229) had two regions with AI, separated by a region of retention.

Thirty of the 50 cases were also analyzed with markers from 3p21.3 (*TTA1*, *CA9*, *CA2*) and from 17p12–p13 (*D17S520*, *D17S513*). Because of a lack of tumor DNA, the remaining 20 cases could not be analyzed with the aforementioned markers; these cases included all of the microdissected ones, thus leaving out the group of diffuse gastric carcinomas from this further analysis. The results are summarized in Table 1. The SRO at 6q and the 17p12–p13 region showed comparable percentages of AI. For the SRO at 6q, the frequency of AI was 35% (8 of 23 informative cases), and for 17p12–p13 this was 38% (8 of 21 informative cases). The frequency of AI at 3p21.3 was 15% (4 of 27 informative cases).

DISCUSSION

We consider the AI observed in this study to represent allelic loss rather than gain of alleles because, in our previous studies, several tumors, including cases 11, 15, and 29, when analyzed with RFLP markers from 6q, showed clear LOH (Queimado et al., 1995; Seruca et al., 1995a); and a cytogenetic analysis of the gastric carcinomas, including cases 29 and 220, also indicated that loss of 6q material is a common abnormality (Seruca et al., 1993).

In a previous study performed by our group (Queimado et al., 1995), an SRO of allelic losses was defined by markers *D6S268* and *ARG1*. In the Cedar database, *ARG1* lies between *D6S407* and

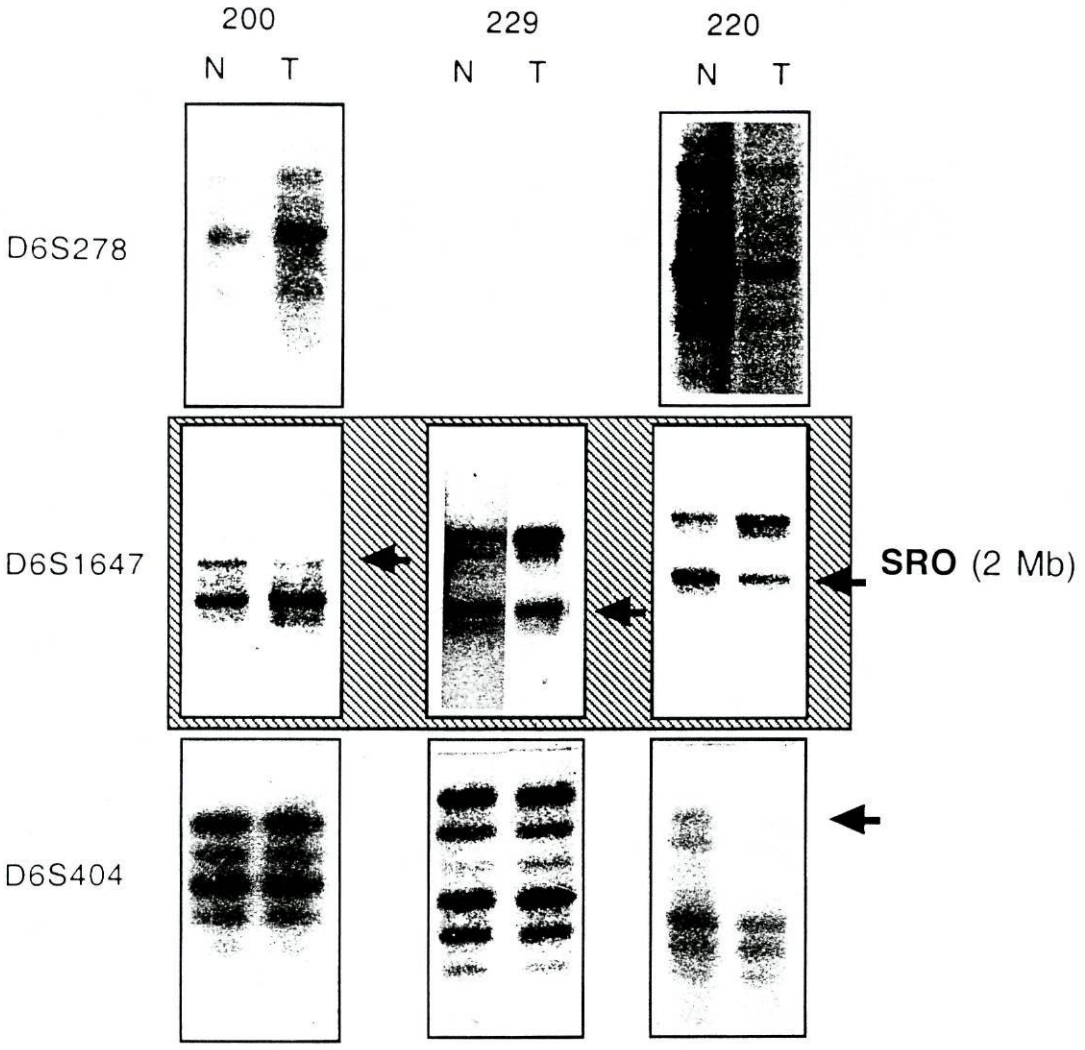

Figure 2. Autoradiograms of cases crucial in delimiting the smallest region of overlap (SRO) between markers *D6S278* and *D6S404*. Allelic imbalance (AI) is indicated by arrows. N, constitutional DNA; T, tumor DNA. Shadowed region, SRO. Case 200 showed AI at *D6S1647* and retained heterozygosity at *D6S278* and *D6S404*. Case 229 showed AI at *D6S1647* and retained heterozygosity at the distal locus *D6S404*. Case 220 showed AI at *D6S1647* and *D6S404* and showed retention at proximal locus *D6S278*.

D6S262. By combining data from several databases, we estimated this SRO to be about 12–16 cM.

In this study, we detected AI in 18 of 50 informative cases (36%). Comparison of the 18 cases that showed AI at one or more loci allowed us to define a new SRO, bordered by the markers *D6S278* and *D6S404*, and within a region with high frequencies of AI (*D6S1647*, 88%; *D6S1698*, 80%). Three cases (200, 220, 229) were crucial for the delineation of this SRO. According to the Cedar database and the Whitehead Institute database (http://www-genome.wi.mit.edu), *D6S278* and *D6S404* are approximately 2 cM apart. Both are in the Whitehead contig WC6.14 and are estimated to be some 3 Mb apart.

For 15 of 32 cases without AI at 6q, we did not obtain information for the defined SRO, either due to uninformativeness at these loci or to the lack of DNA. Thus, allelic loss for this region is not excluded in these 15 tumors. Among the 18 cases in which AI did occur at one or more loci, there were three tumors (cases 11, 209, 231) that did not show AI within the SRO (Fig. 1). Cases 11 and 209 were either not informative or showed retention of heterozygosity at one or more loci within the SRO. For case 231, no information was obtained for this region because of lack of DNA (Fig. 1).

Ten tumors were excluded from AI analysis because of a high frequency of MIN. For three tumors (cases 36, 182, 199), we had already ob-

TABLE 1. Allelic Imbalance (AI) Analysis of 6q16.3–q23.3, 3p21.3, and 17p12–p13[a]

Cases	Histologic type	Tumor stage	Loci		
			6q[b]	3p21.3	17p12–p13
29	Atypical	Early	AI	RET	RET
228	Intestinal	Early	AI	RET	NI
11	Intestinal	Advanced	RET	AI	AI
15	Intestinal	Advanced	AI	AI	RET
149	Intestinal	Advanced	AI	RET	RET
161	Intestinal	Advanced	NI	AI	AI
166	Intestinal	Advanced	AI	RET	AI
175	Intestinal	Advanced	AI	ND	AI
177	Intestinal	Advanced	RET	RET	AI
200	Intestinal	Advanced	AI	RET	RET
224	Intestinal	Advanced	RET	RET	AI
202	Atypical	Advanced	RET	RET	AI
209	Atypical	Advanced	RET	AI	AI
229	Atypical	Advanced	AI	NI	NI

[a]AI, allelic imbalance; RET, retention of heterozygosity; NI, not informative; ND, not determined.
[b]Only the markers *D6S1594*, *D6S1647*, and *D6S1698*, which mapped within the SRO defined in this study, have been taken into account.

served MIN with microsatellites located at other chromosomes (Dos Santos et al., 1996). The overall frequency of MIN tumors in the present series (17%) does not differ greatly from the 9%–25% described previously for gastric tumors with a MIN phenotype (Schneider et al., 1995; Seruca et al., 1995b; Dos Santos et al., 1996; Ottini et al., 1997; Oliveira et al., 1998; Wu et al., 1998). Thus, these 10 cases are likely to represent tumors with a mutator phenotype. In case 36, an *MSH2* mutation was indeed detected (data not shown). An alternative route of gastric carcinogenesis may be involved in these cases.

To assess the importance of the 6q-SRO, we compared the relative frequencies of AI for the three markers that constitute this SRO (namely, *D6S1594*, *D6S1647*, *D6S1698*) with the frequency of AI at two other hotspots of heterozygous deletions in gastric cancer, namely 3p21.3 and 17p13, in a subset of 30 gastric carcinomas. The observed frequencies of AI at 3p21.3 (15%) and 17p12–p13 (38%) are low compared to previous data (Schneider et al., 1995; Seruca et al., 1995a; Gleeson et al., 1997). This is probably due to the number of tumor specimens in which the amount of stromal tissue precludes detection of AI, and also implies that the frequency of LOH at the 6q-SRO may be an underestimate. The data, summarized in Table 1, clearly show that loss at the 6q-SRO is about as frequent as loss at 17p12–p13, and much more frequent than loss at 3p21.3. In addition, two tumors that represent early gastric carcinomas (cases 29, 228) showed LOH at the 6q-SRO and retention of heterozygosity at 3p21.3. This fits the earlier suggestion, by Seruca et al. (1995a), that loss of 6q sequences is an early event preceding loss at 3p21. The high percentage of AI detected at 6q in all histologic types of gastric carcinoma supports the idea that, independent of the histologic differentiation, loss of 6q material is an important, nonrandom event in the early steps of gastric carcinogenesis. For cases with retention of 6q, alternative routes of gastric carcinogenesis may exist. The five tumors with retention at the 6q-SRO in combination with AI at 17p12–p13 and/or 3p21.3 (see Table 1) may constitute a specific subcategory among these cases.

Bands 6q21–q23 are heterozygously lost in several other types of neoplasia, such as breast cancer (Sheng et al., 1996), ovarian cancer (Orphanos et al., 1995), and salivary gland cancer (Queimado et al., 1998). This suggests that the region might harbor one or more tumor suppressor genes of importance not only in the development of gastric cancer, but also in the development of other epithelial tumors. Some candidate tumor suppressor genes have been mapped to this region: *AIM1* (6q21) has been implicated in malignant melanomas (Ray et al., 1996), and *SEN6A* (6q14–q21) induces senescence of ovarian tumor cells (Sandhu et al., 1996). According to the genetic map of Science 98 (http://www.ncbi.nlm.nih.gov/genemap98), *AIM1* maps centromeric to locus *D6S268*, which lies 2 cM proximal to the region defined in the present study. This study thus appears to exclude *AIM1* as a possible candidate gene implicated in gastric tumorigenesis. The *SEN6A* gene has not been identified yet, but could, based on the mapping data, be identical to a gene inducing senescence of BK virus-transformed mouse cells. The latter gene has been mapped to a 4-Mb segment at 6q21 (Morelli et al., 1997). As this 4-Mb segment partly overlaps with the SRO defined in this study, involvement of this gene in gastric carcinoma cannot be excluded.

In summary, apart from confirming that 6q deletions occur in early stages of development and in all histologic types of gastric carcinoma, we have now delimited the 12–16-cM SRO in 6q16.3–q23 to a region of 2 cM and ~3 Mb. This will facilitate the identification of a putative gene in this region, which seems to be involved in a major route of gastric carcinogenesis.

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PAPER II

Allelic Gains and Losses in Distinct Regions of Chromosome 6 in Gastric Carcinoma

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ABSTRACT

In gastric cancer, alterations in the long arm of chromosome 6 are a frequent event. Two regions of heterozygous loss have been described: 6q16.3 - 6q23 and 6q26 - 6q27. We have evaluated by microsatellite - and FISH analyses the 6q status of three cell lines that we established from primary gastric carcinomas xenografted in nude mice, in order to elucidate the underlying mechanisms of 6q alterations. Alterations of the long arm of chromosome 6 were found in all three xenografts and corresponding cell lines. Allelic imbalance was found in the three cases, by microsatellite analysis. FISH analyses clearly demonstrated a duplication of the larger part of the 6q arm in all three cell lines. Two of the cell lines and the corresponding xenografts showed in addition complete loss of one of the parental alleles at the terminal part of 6q. Thus, the allelic imbalances observed along the long arm of chromosome 6 are representing gain of alleles in one distinct chromosomal segment and loss of alleles in another distinct segment.

INTRODUCTION

As in other types of tumors, gastric carcinomas result from the accumulation of several genetic alterations. These alterations may fall into two main pathways of development of cancer (Lengauer et al., 1998). One is the hypermutability pathway where inactivation of repair genes (MSI phenotype) leads to an increase of the mutation rate, affecting many different genes (Oliveira et al., 1998) and resulting in a deregulated proliferation of cancer cells. The other is the chromosomal instability pathway that is characterized by gross chromosomal alterations resulting in aneuploidy of cancer cells and leading to inactivation of tumor suppressor genes and activation of oncogenes (Dos Santos and van Kessel, 1999).

Several studies have pinpointed chromosome 6 as a target of chromosome instability in the development of gastric cancer. Karyotypically detectable deletions of the long arm of chromosome 6, always involving region 6q21-q22 -> qter, have been observed in 26-45% of primary gastric carcinomas (Ochi et al., 1986; Seruca et al., 1993; Panani et al., 1995). Restriction fragment length polymorphism (RFLP) analysis also showed that the long arm of chromosome 6 is frequently deleted in gastric carcinomas (Seruca et al., 1995; Gleeson et al., 1997). Two regions of heterozygous loss on 6q have been described in primary sporadic gastric carcinomas: one interstitial, between 6q16.3 and 6q23, is lost in 50% of informative cases; the other distal, at 6q26-q27, is lost in 37% of informative cases (Queimado et al., 1995). Recently, we delimited the interstitial region to a 2 cM segment, flanked by loci *D6S278* and *D6S404* (6q16.3-q23.2) (Carvalho et al., 1999).

Prominent problems in loss of heterozygosity (LOH) studies in primary tumors are admixture of stromal cells and presence of clonal heterogeneity. In xenografts and/or cell lines the problem of admixed normal tissue does not occur. Moreover, in the xenografts and/or cell lines, there is a positive selection for clones with a higher growth potential so that information can be obtained on the role of genetic alterations in tumor development and

progression. Therefore, we studied chromosome 6 alterations in three cell lines derived from primary gastric tumors that had been xenografted in nude mice. Data on the pathologic characteristics of two of these cell lines have previously been described (Gärtner et al., 1996). Allelic imbalances (AI) of the cell lines and the respective primary and xenografted tumors were analyzed with microsatellite markers. Results were compared with those from FISH analysis of metaphases of the cell lines in order to elucidate the underlying mechanisms.

MATERIALS AND METHODS

Xenografts

Specimens obtained from primary and metastatic sites of eight human gastric cancers were partly used to make formalin-fixed paraffin-embedded specimens for pathology examination and partly cut in pieces with 5-6 mm diameter at the time of surgery. These pieces were transplanted subcutaneously in the inter-scapular region of male and female nude mice (athymic N: NIH(s)II-*nu/nu* nude mice 4-6 week old). Tumoral growth was monitored once a week by ruler measurement of tumor length (a) and width (b). Tumor volumes were estimated by the formula – $V(mm^3) = a \times b^2 \times 1/2$. Tumor doubling times (Td) were calculated from a plot of tumor volumes *versus* days after implantation. Tumorigenicity was determined 6-8 months after transplantation by observing whether or not tumor growth had occurred. When the tumor volume reached approximately 1500 mm^3, the tumor was resected and divided into small fragments 5-6 mm diameter. One fragment was then reimplanted into each of five nude mice to preserve the transplantable tumor line.

From the eight gastric carcinomas that were heterotransplanted into nude mice, five achieved growth. Attempts to culture these xenografts *in vitro* were successful in three cases, resulting in three cell lines (GP 195, GP 202 and GP 220).

According to Lauren's classification (Laurén, 1965), the primary tumors of cases 195 and 220 were of the diffuse type. The primary tumor of case 202 was an atypical type tumor with a component of diffuse cells. After transplantation of the primary tumor of case 202 only diffuse cells were observed in the xenograft.

Microsatellite analysis

The sixteen polymorphic microsatellite markers used were *D6S305* and *D6S281* (Gyapay et al, 1994), *TBP* and *D6S252* (http://cedar.genetics.soton.ac.uk/pub) plus the twelve used in our

previous study (Carvalho et al., 1999). Of the sixteen, 12 mapped to the region 6q16.3-q23.3, one mapped at 6q14.2-q16.2 and three mapped at 6q27 (Figure 1). Microsatellite analysis was performed as previously described by Carvalho et al (1999). All analyses were carried out at least *in duplo*. Signal intensities were determined either by densitometry on an Ultrascan XL LKB densitometer using Gel Scan XL 2.1 software (Pharmacia LKB), or on a phosphor-imaging system (STORM 860, Molecular Dynamics).

FISH analysis

FISH analysis of the three cell lines (GP195, GP202, GP220) was performed according to routine procedures with the following probes: a) a Bio-11-dUTP-labeled chromosome 6-specific library (wcp6); b) a digoxigenin-11-dUTP labeled-chromosome 6 centromeric probe (p308), in combination with Bio-11-dUTP-labeled CEPH YAC 870B3 (6q16.3-q21), and c) CEPH YACs 801C2 (6q21-q23.3) and 820F9 (6q27) labeled with digoxigenin-11-dUTP and Bio-11-dUTP, respectively, or d) a digoxigenin-11-dUTP labeled-chromosome 6 centromeric probe (p308), in combination with Bio-11-dUTP-labeled CEPH YAC 820F9 (6q27). The position of the YACs relative to the microsattelite markers is indicated in Figure 1. Chromosomes were counterstained with DAPI. For each analysis 25 metaphases were analyzed.

RESULTS

Microsatellite analysis

Figure 1 shows the results of the microsatellite analysis of the three cell lines (GP195, GP202, GP220) and the respective primary and xenografted tumors. No allelic imbalances were observed in the primary tumors of cases 195 and 202. Xenografts and cell lines of these two cases, however, showed allelic imbalances for all informative loci between *D6S252* and *D6S305*. Complete loss of one parental allele was detected for case 195 at *D6S281* and *TBP*, and for case 202 at *D6S305*, *D6S281* and *TBP*.

Using DNA extracted from frozen material of the primary tumor of case 220, allelic imbalances could not be detected. However, when tumor cells of case 220 were carefully microdissected from paraffin sections, allelic imbalance was detected for both markers that could be analyzed, namely *D6S1647* and *D6S404*. The xenograft and the cell line of case 220 showed AI for all informative loci on chromosome 6q, but without complete loss of one of the parental alleles.

For those loci where visual inspection of the autoradiograms indicated an allelic imbalance, the ratio between the alleles was quantified. The ratios varied between 1.2 and 2.2 (Figure 1). For the same markers, densitometric scanning of the autoradiograms appeared to result on average in lower ratios than analysis by the phosphor-imager system (see Figure 1, case 220).

FISH analysis

All three cell lines were successively hybridized with a chromosome 6-specific library (wcp6), with Y870B3 from 6q16.3-q21 in combination with a chromosome 6-specific centromeric probe, and with Y820F9 from 6q27 in combination with either the centromeric probe or Y801C2 from 6q21-q23.3.

When hybridized with the chromosome-specific library, cell line GP195 showed two apparently normal chromosomes 6 (with respect to size as well as banding) and one derivative chromosome 6 that appeared larger than a normal chromosome 6, but nonetheless consisted entirely of chromosome 6 material (Figure 2A). The centromeric probe and the proximal YAC (Y870B3) both hybridized to the same set of three chromosomes (Figure 2B). Also the middle YAC (Y801C2) hybridized to three chromosomes (not shown). The distal YAC (Y820F9), however, hybridized to only two of these three chromosomes (Figure 2C). All metaphases of GP202 contained one apparently normal chromosome 6 and two translocation products that consisted only in part of chromosome 6 material (Figure 2A). The centromeric probe gave a signal on two chromosomes; Y870B3 gave a signal on the same two chromosomes and on one other chromosome (Figure 2B). Y801C2 gave a signal on three chromosomes, and Y820F9 on two of these three chromosomes (Figure 2D). Thus, like GP195, GP202 appears to contain three 6q arms, one of which is having a terminal deletion. GP220 contained one apparently normal chromosome 6 and three translocation products that consisted in part of chromosome 6 material (Figure 2A). The centromere 6-specific probe gave a signal on two chromosomes, one of which also had a signal for Y870B3. In addition, this YAC gave a signal on two other chromosomes(Figure 2B). Y801C2 and Y820F9 also gave signals on three chromosomes (Figure 2D). GP220 clearly has three 6q arms. A schematic representation of the FISH results is shown in Figure 3.

DISCUSSION

We have established cell lines from three primary gastric carcinomas previously xenografted in nude mice. Since primary sporadic gastric carcinomas frequently show alterations in the long arm of chromosome 6 regardless of their histologic differentiation (Ochi et al., 1986; Seruca et al., 1993; Seruca et al., 1995; Gleeson et al., 1997), also in the early steps of tumor development (Seruca et al., 1995), we focused in the present study on the evaluation of the 6q status of these cell lines and the xenografts from which they originated.

Alterations of chromosome 6 were found in all three xenografts and corresponding cell lines. A large part of the long arm of chromosome 6 appeared *in triplo* in all cell lines, presumably as a result of a partial chromosome duplication. For GP195 the duplication included the centromere. GP 195 and GP 202, and the corresponding xenografts, appeared to have lost one of the parental regions 6q27-qter, which is seen as a complete loss of heterozygosity of this segment. This loss of heterozygosity may have preceded the duplication event. None of these alterations were detected in the primary tumors. Only for one tumor (case 220) where microdissected material was available, we could detect allelic imbalance in the primary tumor.

The seeming discrepancy between the findings in the primary tumors on one hand, and those in the xenografts and cell lines on the other hand, may be explained by a masking of the allelic imbalance in the primary tumors as a consequence of a large admixture of normal cells. This may in particular occur in diffuse type carcinomas, such as the primary tumor of case 220. When tumor sections of this case were very carefully microdissected, the allelic imbalance present in the xenograft could also be detected in the primary tumor. Another explanation may be that the primary tumors are heterogeneous with respect to their genomic alterations, and that clonal selection has occurred during the growth of the xenograft. Case 202 may be an example of this. The primary tumor of this case was classified as atypical with

a component of diffuse cells, whereas in the matching xenograft only diffuse type cells were observed. Microsatellite analysis by itself cannot distinguish between these two possibilities. Moreover, both may be true. (both may occur)

The FISH analyses clearly demonstrated that the three cell lines are homogeneous with respect to their chromosome 6 content, since subclonal variations did not occur in the 25 metaphases analyzed per case. The FISH data indicated a duplication of the region 6q16.3-q23.3 in all three cell lines. In the microsatellite analysis this should have resulted in a ratio of 1:2 between the alleles. In some cases, however, the ratio between the alleles, as determined by densitometric analysis, strongly deviated from this expected value. The quantitative data obtained with the phosphor-imager were more in agreement with the FISH data, but some markers still gave a marked underestimation of the allele ratio, as shown in case 220 (Figure 1). This indicates that the ratio that is obtained with this type of semi-quantitative analysis may vary for different markers and/or primer sets, irrespective of the actual situation in the tumor.

In a recent analysis of 60 primary gastric carcinomas AI was interpreted as loss of one of the parental alleles and not as a gain of 6q material, and we defined a 2-cM smallest region of overlap of heterozygous deletions (Carvalho et al., 1999). The question needs to be addressed whether this interpretation is still correct. One conclusion from the present work is that one should be cautious to interpret allelic imbalance with allele ratios lower than 2 as the result of heterozygous deletions. As clearly shown by our FISH results, allele ratios lower than 2 may also result from aneuploidy of the analyzed loci. However, the three cell lines, and most likely also the three matching xenografts described here, all had a duplication of the q-arm of one of the parental chromosomes 6. In primary gastric carcinomas, the situation is noticeably different. Here, we often detected allelic imbalance for a small region of 6q that was flanked by regions of retention (Carvalho et al., 1999). Such a pattern may represent true

loss rather than a duplication of only a small segment of the q-arm. This interpretation was supported by the fact that some of the samples had previously shown loss of heterozygosity with RFLP markers from the same region (Queimado et al., 1995).

The overrepresentation of the long arm of chromosome 6 observed in the three cell lines may indicate that during the in vivo and in vitro culture, clones with this region in triplicate, undetectable in the primary tumors, had a selective advantage. Though the number of cell lines is limited, this consistent overrepresentation of the 6q16.3-q23.3 region, particularly in the near-diploid cell line GP220, suggests the presence of (a) proliferative gene(s). An overrepresentation of part of this region, namely 6q21-q22.2, in combination with a distal deletion region has also been reported for one of five breast cancer cell lines that were similarly analyzed (Zhang et al., 1998). On the other hand, two of our cell lines, GP202 and GP195 (Gärtner et al, 1996 and Figure 2) have a widespread overrepresentation of chromosomes. Thus, at least for these two cell lines it cannot be ruled out that the observed gain of 6q material is part of a general increase of ploidy of the cells rather than an isolated event related to tumor development or progression.

Two cases (195 and 202) show complete loss of one of the parental alleles at the terminal part of the long arm of chromosome 6. The terminal 6q region was already pinpointed by Queimado et al. (1995) as being frequently lost in gastric carcinoma, and may therefore harbor a tumor suppressor gene. The availability of two gastric cancer-derived cell lines with a terminal deletion of 6q may be useful for further –functional- studies aiming at the identification of the putative tumor suppressor gene(s) in this region of 6q.

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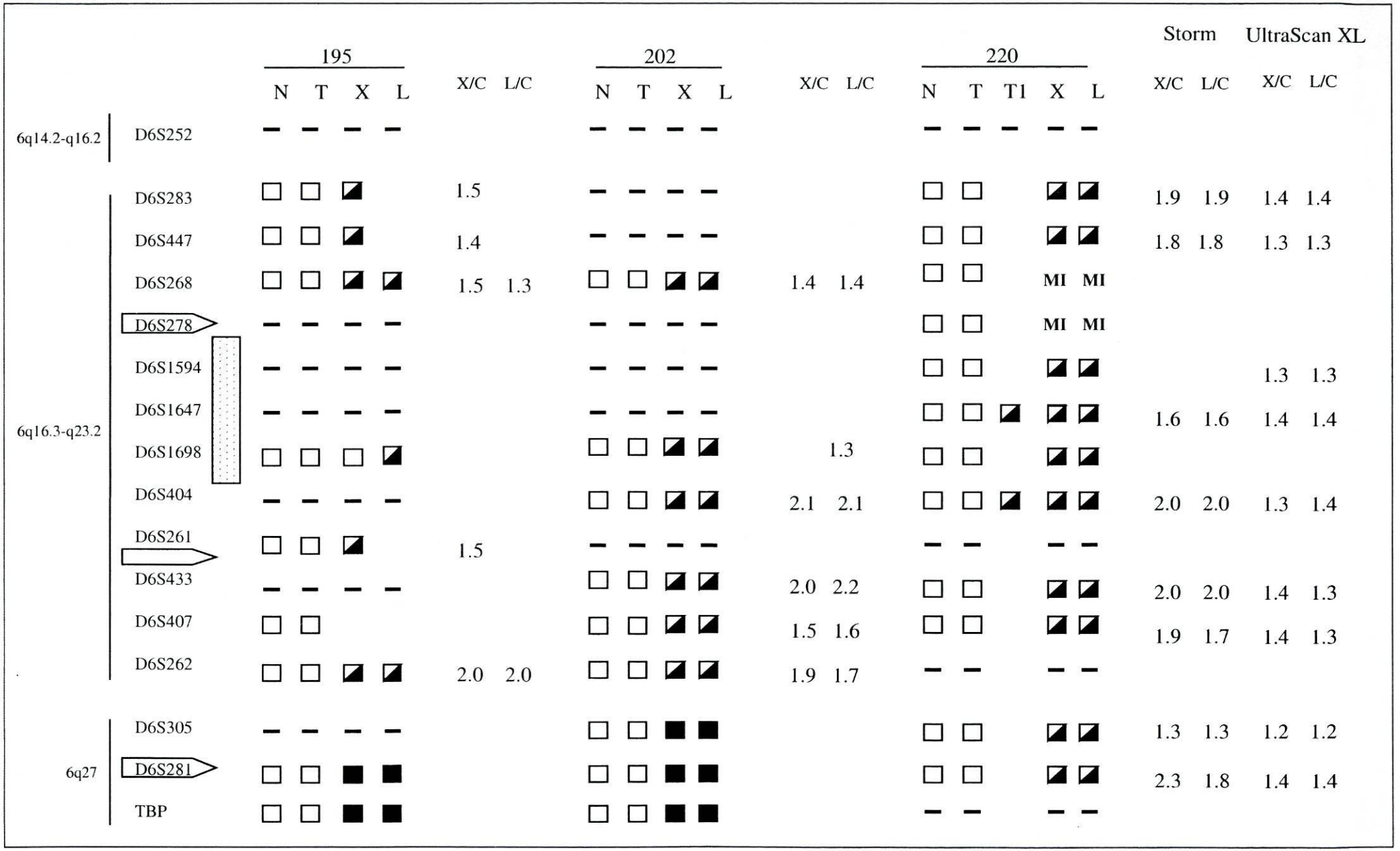
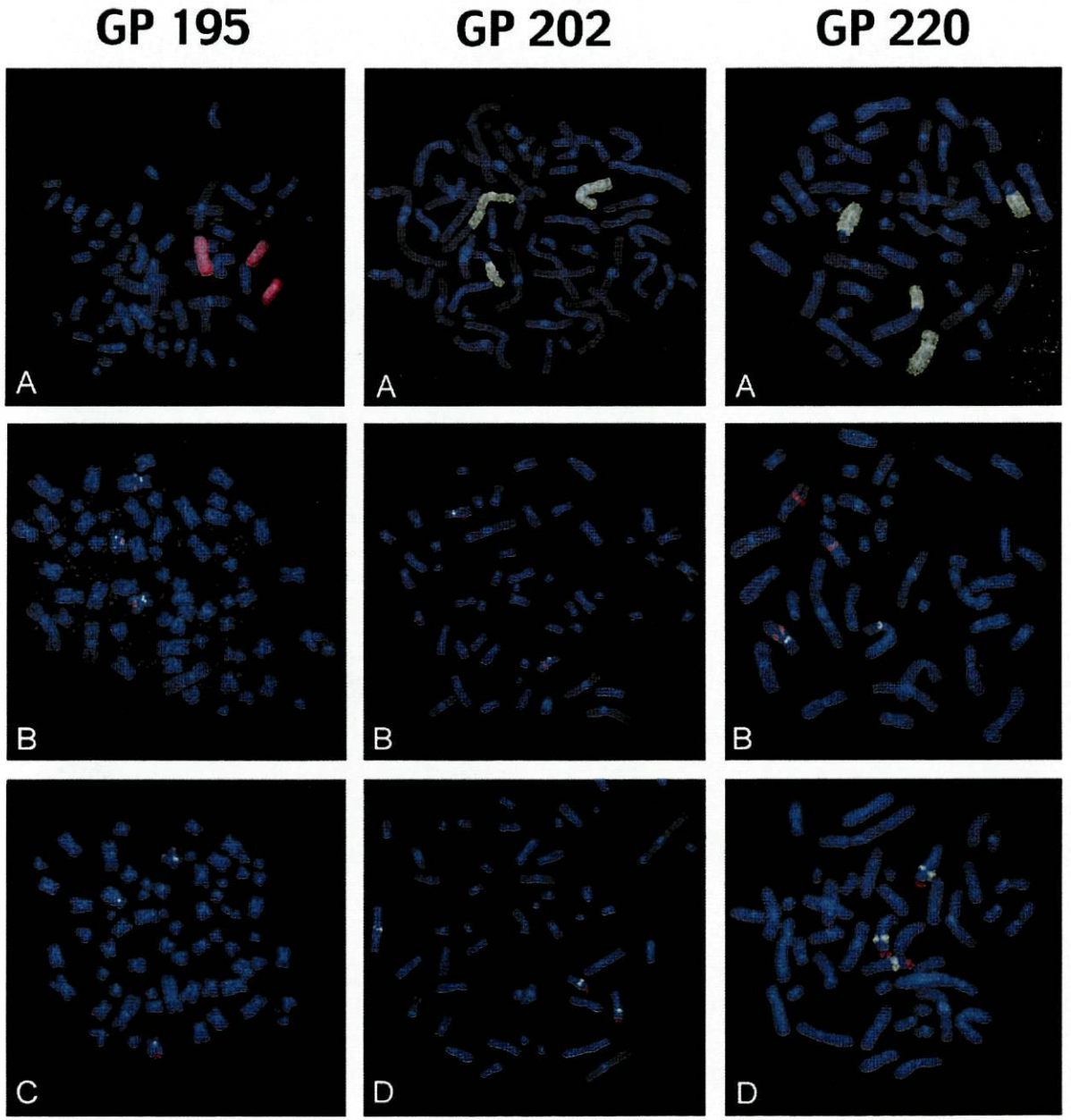
FIGURE LEGENDS

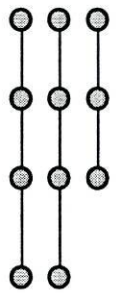
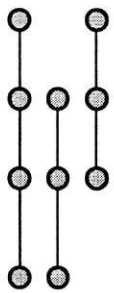
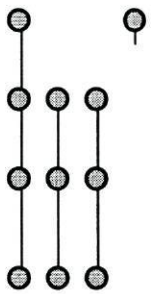
Figure 1. Microsatellite analysis of cases GP195, GP202 and GP220. N, normal mucosa; T, primary tumor; T1, microdissected primary tumor; X, xenograft in nude mice; L, cell line; X/C and L/C, allelic ratios for xenografts and cell lines, respectively. □, retention of heterozygosity; ◪, allelic imbalance; ■, LOH; -, not informative; MI, microsatellite instability. The approximate position of the YACs used in the FISH analysis (see Figure 2) are indicated the open arrows on the left. The grey bar indicates the smallest region of overlap of heterozygous deletions as defined previously (Carvalho et al., 1999).

Figure 2. FISH analysis of cell lines GP195, GP202 and GP220. Panels A, Chromosome 6 painting probe (wcp6); panels B, Bicolor FISH with a chromosome 6 centromere probe (green signal) and Y870B3 (red signal); panel C, Bicolor FISH with Y801C2 (green signal) and Y820F9 (red signal); panels D, Bicolor FISH with a chromosome 6 centromere probe (green signal) and Y820F9 (red signal).

Figure 3. Schematic representation of the FISH results. Hybridization signals that appear on the same chromosome are vertically aligned.


195
202
220
Storm
UltraScan XL
N T X L X/C L/C
N T X L X/C L/C
N T T1 X L X/C L/C X/C L/C
6q14.2-q16.2
D6S252
D6S283 1.5 1.9 1.9 1.4 1.4
D6S447 1.4 1.8 1.8 1.3 1.3
D6S268 1.5 1.3 1.4 1.4 MI MI
D6S278 MI MI
D6S1594 1.3 1.3
D6S1647 1.6 1.6 1.4 1.4
6q16.3-q23.2
D6S1698 1.3
D6S404 2.1 2.1 2.0 2.0 1.3 1.4
D6S261 1.5
D6S433 2.0 2.2 2.0 2.0 1.4 1.3
D6S407 1.5 1.6 1.9 1.7 1.4 1.3
D6S262 2.0 2.0 1.9 1.7
D6S305 1.3 1.3 1.2 1.2
6q27
D6S281 2.3 1.8 1.4 1.4
TBP


GP 195
GP 202
GP 220
A
A
A
B
B
B
C
D
D

	GP 195	GP 202	GP 220
Cen6 Y870B3 Y801C2 Y820F9			

PAPER III

Identification of expressed sequences from yeast artificial chromosomes by means of suppression subtractive hybridisation

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ABSTRACT

In our search for genomic regions that are involved in the development of gastric cancer, we recently identified a 2 cM minimal region of overlapping heterozygous deletions in 6q16.3-q23.1 (1). In this study we describe an application of the suppression subtraction method developed by Diatchenko et al (2) to search for genes in a small region of the genome, taking advantage from the fact that many human genes present on yeast artificial chromosomes are expressed in yeast. Subtraction was performed with two non-overlapping YACs that covered a region of approx. 2.3 Mb. Combined forward and reversed subtractions resulted in the identification of 12 clones of human origin, 8 of which could be confirmed as originating from 6q. The whole procedure, including mRNA isolation and differential screening, could be performed in only two weeks, clearly demonstrating the strength of this method to identify novel transcripts in Mb-size sections of the human genome.

INTRODUCTION

In our search for genomic regions that are involved in the development of gastric cancer, we recently identified a 2cM minimal region of overlap of heterozygous deletions in 6q16.3-q23.1 (1). Several methods are available to initiate a search for genes in such a limited genomic region, such as direct screening of cDNA libraries, cDNA selection-PCR based methods, cross-species sequence homology searches, screening of CpG islands, exon trapping strategies or screening for splice sites (for review see 3). Though all these methods can be useful in the identification of genes, some are very laborious and time-consuming. There is also a variety of methods allowing identification of differentially expressed genes, such as cDNA subtractive hybridisation (SH) (for a review see 4), differential display (DD) (5), representative difference analysis (RDA) (6), serial analysis of gene expression (SAGE) (7) and suppression subtractive hybridisation (SSH) (2). These latter methods, however, analyse complete genomes instead of restricted regions. In this study, we describe application of a subtraction method to analyse only a small region of the genome. Our strategy took advantage from the fact that many human genes present on yeast artificial chromosomes are expressed in yeast (8). Thus, in a cDNA subtraction protocol using two yeast clones that contain different YACs, the resulting pool of cDNAs will be enriched for genes expressing themselves on the human inserts of the YACs. Among the various subtraction methods available, we chose the suppression subtractive hybridisation strategy (2, 9) to analyse the gene contents of two YACs from the 2 cM smallest region of overlap of heterozygous deletions in gastric cancer.

MATERIAL AND METHODS

Isolation of RNA and DNA from yeast

Yeast cells containing different CEPH YACs were grown for 48h at 30 °C. Total RNA was isolated according to the RNAzol™ B procedure (Campro Scientific). Poly A^+ RNA was obtained using the mRNA purification kit from Amersham Pharmacia Biotech. DNA was isolated by standard salt-chloroform extraction (10).

Suppression subtractive hybridisation

First strand cDNA was synthesized from 2 μg of poly A^+ RNA using AMV reverse transcriptase from Clontech's PCR-Select™ cDNA subtraction kit. Subtraction was performed according to the instructions of the kit (PCR-Select™ cDNA Subtraction kit, Clontech), but with the following modification. Diluted cDNAs (subtracted and unsubtracted samples) were further diluted 50x before the primary PCR. PCR products were subcloned into pCR™II using a TA Cloning kit (Invitrogen). The transformed bacteria were plated on selective plates and white colonies were selected randomly, resulting in two cDNA libraries, specific for the forward and reverse subtractions, respectively.

Subtraction efficiency

Primers for the yeast housekeeping gene ZWF1 (encoding glucose-6-phosphate dehydrogenase, accession nr. M34709) were designed in order to check the depletion of non-differentially expressed sequences. Primer sequences were: forward 5'-GGATTCCAGAGGCTTACGAG-3', reverse 5'-GGGTGCTTTTCGGGCATAAC-3', resulting in an amplicon of 232 bp. Amplification was for 30 cycles with denaturation at 94 °C for 0.5 min, annealing at 58 °C for 0.5 min, and extension at 72 °C for 2 min. The initial denaturation step at 94 °C and the final extension at 72 °C lasted for 3 min.

Differential screening

Inserts of the selected colonies were amplified by touch–PCR. The PCR was carried out in a 25-µl reaction volume containing 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM $MgCl_2$, 200 mM of each deoxynucleotide triphosphate, 0.4 mM of each primer (M13 forward 5'-GTAAAACGACGGCCAG-3'; M13 reverse 5'-CAGGAAACAGCTATGAC-3'), and 0.5 U of Taq DNA polymerase (Pharmacia). Amplification was for 30 cycles with denaturation at 94°C for 0.5 min, annealing at 50°C for 0.5 min, and extension at 72°C for 1.5 min. The initial denaturation step at 94°C and the final extension step at 72°C lasted for 3 min. Part of the PCR product (7 ul) was analysed on a 2% agarose gel. PCR products were denatured by addition of an equal volume of 0.5M NaOH, and 3-µl aliquots were dot-blotted on nylon membranes (Hybond-N+; Amersham Pharmacia Biotech). Of each filter, four identical copies were made. DNA was cross-linked to the membranes by incubation at 80 °C for 2 h. Membranes were hybridised with both non-cloned subtracted cDNA pools. cDNA clones that hybridised predominantly with the cDNA pool from which they originated, were considered specific for that cDNA pool.

Southern analysis and Northern analysis

Inserts from the selected clones were hybridised to Southern membranes containing *Eco*RI digests of human DNA, DNA from the two YACs used in the subtraction (Y776A5, Y785D2) and from YACs overlapping with them (Y798G12, Y911F5). DNA from YAC 915E4, mapping to distal 6q was used as negative control. The inserts were labelled with 30 µCi [α-^{32}P] dCTP by random primer labelling. Probes and Southern membranes were prehybridised with 10 µg and 15 µg human Cot-1 DNA (GibcoBRL), respectively. Hybridisation was overnight at 65 °C in a buffer containing 0.5M NaHPO4 (pH 7.0), 7% SDS, and 1mM EDTA. For washings, solutions of decreasing stringencies (2x SSC/0.1% SDS; 1x SSC/0.1% SDS; 0.3x SSC/0.1% SDS; 0.1x SSC/0.1% SDS) were used at 65 °C. cDNA clones were hybridised

to commercial available multiple-tissue Northern blots (#7759-1; #7767-1; #7780-1 - Clontech), following the supplier's instructions.

Sequencing analysis

Selected clones were sequenced using an ABI Prism ™377 DNA Sequencer (Perkin Elmer) and M13 primers from the pCR™ vector. The cDNA sequences were compared to the available databases using BLAST search (http://www.ncbi.nlm.nih.gov/blast/blast.cgi).

RESULTS

From the Whitehead Institute database, 4 YACs were selected that according to their STS marker content mapped within the 2-cM smallest region of overlap (SRO) of heterozygous deletions (1). YAC 776A5 (990 kb) contains marker D6S278 flanking the SRO on the proximal side and markers D6S1594 and WI1240 that map within the SRO. YAC 785D2 (1370 kb) contains the markers WI3490 and D6S1647 that also map within the SRO. The STS content of each YAC was confirmed by PCR (data not shown). YACs were tested by FISH to metaphases to be nonchimeric. The relative position of the YACs as depicted in Figure 1, is based on their STS content.

cDNA subtraction and differential screening

The two contiguous but non-overlapping YACs 776A5 and 785D2 were used for the subtraction. Forward and reverse subtractions were performed in parallel, with Y776A5 as tester in the forward subtraction and Y785D2 as tester in the reverse subtraction. The efficiency of the subtraction was assessed by determining the abundance of the yeast ZWF1 gene, a housekeeping gene, in the cDNA pools. When the unsubtracted and the subtracted cDNA pools were subjected to PCR using ZWF1 gene-specific primers, a PCR product with the expected length was obtained only for the unsubtracted cDNA pool (Figure 2). This indicates that the subtraction step had indeed resulted in depletion of the yeast ZWF1 gene. After subcloning of the subtracted cDNA populations, two libraries of 192 cDNA clones each were generated, corresponding to the forward and the reverse subtraction, respectively. Of these 384 clones, 7 had no insert. The inserts of the other clones as determined by touch-PCR varied in length between 100 bp and 800 bp (Figure 3).

In the differential screening, the membranes that carried the dot-blotted inserts of the cloned cDNAs were subsequently hybridised with the two cDNA pools resulting from the forward and reversed subtractions. Clones were considered specific for the cDNA pool from which

they were derived, when a strong hybridisation signal with that cDNA pool was observed in combination with absence of a signal or a very faint hybridisation signal with the other cDNA pool (Figure 4). Inserts from the clones selected by the differential screening were hybridised to the dot-blotted cDNA libraries to identify identical clones. Thus, the 30 clones that were selected for further analysis appeared to represent 21 independent clones (Tables 1 and 2)

Analysis of the selected clones

All selected clones were hybridised to *EcoR1*-digested human placenta DNA, to *EcoR1*-digested DNA from the YACs that were used in the subtraction, and to *EcoR1*-digested DNA from two other YACs that were overlapping with them. An example of such an analysis is shown in Figure 5. Hybridisation of clone 180, selected from the forward subtraction, to human DNA resulted in a single hybridising band of approximately 9 kb. When the same clone was hybridised to the two YACs used in the subtraction, a single band of the same size as for human DNA was detected in the tester YAC (Y776A5), but not in the driver YAC (Y785D2). A band with the same size was also observed in Y798G12 that partly overlaps with Y776A5. Data from the Southern analysis of the selected clones are summarised in Table 1 for the forward subtraction and in Table 2 for the reverse subtraction. In total, four out of seven clones of the forward subtraction gave rise to a unique band when hybridised to human DNA and to a band of similar size when hybridised with the tester YAC (Y776A5). The remaining three clones did not give a signal with human DNA, but gave specific and identical bands with all YACs.

In the reverse subtraction, 8 out of 14 clones gave rise to a unique band when hybridised to human DNA. All these 8 clones gave a band of similar size when hybridised with the tester YAC (Y785D2). For the remaining 6 clones a specific signal was seen in the hybridisation to all YACs, but not in the hybridisation to human DNA.

Hybridisation of the clones to the set of four YACs gave us some information about the position of these clones within the YAC contig. A schematic representation of the physical location of the cDNAs is depicted in Figure 1.

Comparison of the sequences of the 12 human clones to the BLAST database did not reveal any homology to known genes. However, 8 out of the 12 clones did show a high degree of homology with genomic sequences from 6q.

As a first approach to verify that the selected clones indeed represented sequences that are transcribed in – a subset of - human tissues, we hybridised their inserts to multiple-tissue Northern blots. From the 11 clones that were analysed, clone 5 from the first subtraction and clones 17, 132 and 144 from the second subtraction, showed expression in normal tissues of different embryological origin. An example is shown in Figure 6.

DISCUSSION

Our previous study on allelic imbalances in 60 gastric carcinomas with 19 microsatellite markers predominantly from 6q16-q23, resulted in the identification of a smallest region of overlap of heterozygous deletions of approx. 2 cM (1). Instead of trying to narrow down the region by increasing the number of tumours or by including more markers, we decided to assemble a YAC contig for this region as starting point for a gene search. Several methods have been developed to identify genes in large segments of genomic DNA. Direct screening of cDNA libraries with whole YACs can be successful, but is dependent on the expression of the gene within the cDNA library used (11, 12, 13). Exon trapping has also been used successfully, but is a time-consuming procedure known to often generate many false-positives. In general, cosmid-size fragments are used as starting material. That would have urged us to construct a cosmid contig of the region of interest.

Reports that human and mouse genes encoded on YACs are transcribed in yeast (8, 14, 15) suggest that these genes could in principle be identified by methods that analyse differentially expressed genes. By using two yeast clones, containing different YACs in a subtraction protocol, it should be possible to specifically isolate the transcribed sequences that are encoded by these YACs. Thus, a subtraction method could be used to identify unknown genes in a limited region of the genome.

Diatchenko et al (2) have developed a PCR-based cDNA subtraction technique (SSH - suppression subtractive hybridisation) that has some advantages compared to other subtraction methods. (a) It equalises transcripts abundance among the target cDNA population, which allows the detection of rare transcripts. (b) Only one round of subtraction is needed, and (c) due to the suppression effect during the PCR, physical separation of the unhybridised cDNAs (differentially expressed transcripts) is not necessary. Diatchenko et al (2) used this method to subtract human testis cDNAs against a mixture of cDNA derived from 10 different human tissues and achieved a high level of enrichment of testis-specific cDNAs.

They used the subtracted cDNA mixture to screen a cosmid library constructed from flow-sorted human Y chromosomes. By a subsequent Northern analysis with 37 selected cosmids 5 different chromosome Y-specific genes expressed in testis were identified. The application of this method (16) also resulted in the identification of 27 genes differentially expressed to an estrogen receptor-positive breast carcinoma cell line and an estrogen receptor negative breast carcinoma cell line.

Here, we have applied this subtraction approach to two non-overlapping YACs that covered a region of approx. 2.3 Mb. The combined forward and reversed subtractions resulted in the identification of 21 unique clones. Of these, 12 appeared to be of human origin, as they hybridised to human DNA and to the tester YAC. The remaining 9 clones hybridised to all yeast DNA samples present on the membrane, but not to human DNA. These clones presumably represent yeast cDNAs. This conclusion was confirmed by sequence analysis of some of these clones (data not shown), that indicated a high degree of homology with yeast sequences. The detection of yeast cDNAs may be caused by the differential expression of some yeast genes between the two cultures or may be intrinsic to the method that only guarantees an enrichment in differentially expressed genes. The efficiency of the subtraction was indicated by the fact that transcripts of the yeast housekeeping gene ZWF1 could no longer be detected in the subtracted cDNA pool.

The isolation of 12 transcribed fragments of human origin already demonstrates the strength of this method. We tested how many of these fragments indeed represented human genes by sequence analysis of these clones and by Northern analysis. Four out of the 11 clones that were subjected to a Northern analysis showed expression in various tissues. Clone 24 (Y785D2) was not subjected to Northern analysis, since the comparison of its sequence with the database showed a high degree of homology with LTR7–HERVH elements. Our failure to detect expression for 7 clones can be caused by an overall low expression in human tissues in combination with the small size of the clones. Alternatively, some of the clones may

consist of intronic sequences that were not spliced out during the mRNA maturation process. Splicing of foreign transcripts in yeast may not be efficient (17). Human and mouse sequences do not contain some of the consensus sequences needed by the splicing machinery in yeast to properly excise introns (18, 14).

Summing up, the strategy used in this work resulted in the identification of 12 cDNA clones that map to the human DNA segment covered by the YACs 776A5 and 785D2. The fact that the whole procedure, from mRNA isolation until differential screening of the dot-blots, can be done in only 12 days, clearly demonstrates the strength of this method to identify novel transcripts in Mb-size sections of the human genome.

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Figure 1. Schematic representation of the YAC contig in the 6q region and location of the human clones relative to the YACs.

Figure 2. Results from the amplification of ZWF1 gene on the subtraction of YACs 776A5 and 785D2. Lanes 1 and 2, unsubtracted cDNA from 776A5 (tester) and 785D2 (tester), respectively; lanes 3 and 4, Subtracted cDNA from 776A5 (tester) and 785D2 (tester), respectively; lane 5 genomic DNA from YAC 785D2 (control of PCR).

Figure 3. PCR products of clones from the cDNA library. Insert sizes vary from 100bp to 800bp.

Figure 4. Example of a result from differential screening hybridisation. Hybridisation of the cDNA library of clones specific for Y785D2 (reverse experiment of the first subtraction. A, probe: pool of cDNAs subtracted (not cloned) from the reverse experiment of the first subtraction (Y785D2 as tester); B, probe: pool of cDNAs subtracted (not cloned) from the forward experiment of the first subtraction (Y776A5 as tester). Examples of some positive clones specific for Y785D2 (clones 8, 11, 24, 44) are indicated with a circle.

Figure 5. Southern analysis. Human clone - 180 (Y776A5)- 9 kb. Yeast clone – 146 (Y785D2). Lane 1, Y915E4 (6q27); lane 2, Y776A5; lane 3, Y785D2; lanes 4 and 5, human placenta; lane 6, Y798G12; lane 7,Y911F5.

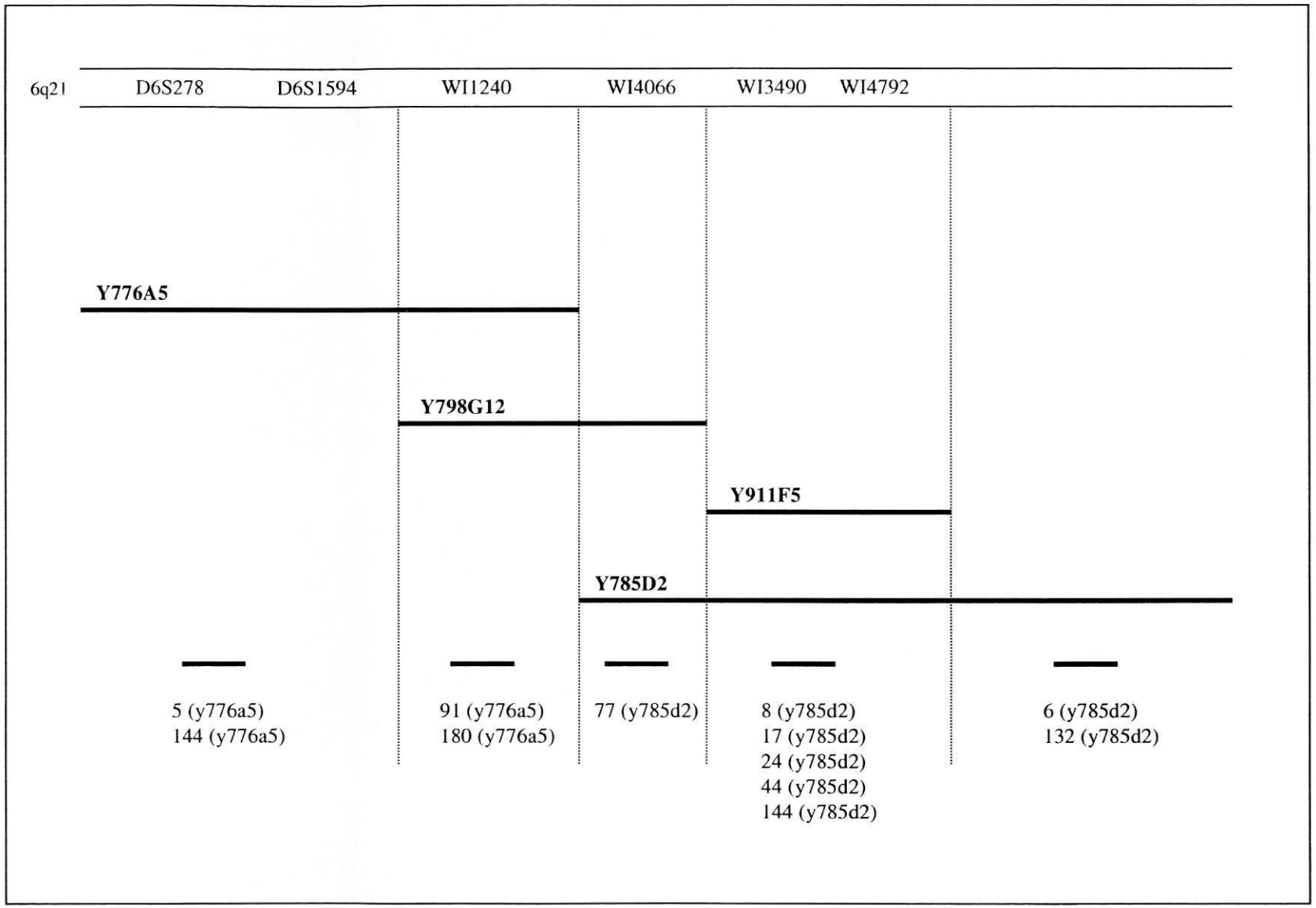
Figure 6. Northern analysis. Clone 144 (Y785D2). Lane 1, stomach; lane 2, thyroid; lane 3, spinal cord; lane 4, lymph node; lane 5, trachea; lane 6, adrenal gland; lane 7, bone marrow.

Table 1. Southern analysis data of selected clones from the forward subtraction (tester Y776A5)

Clones	Size (bp)	Human placenta	Size (kb)	Y776a5	Y785d2	Y798g12	Y911f5	Y915e4	Similar clones
5	364	+	5.0	+	-	-	-	-	n = 5
26	-	-	-	+	+	+	+	+	-
52	-	-	-	+	+	+	+	+	-
63	-	-	-	+	+	+	+	+	-
91	584	+	12.0	+	-	+	-	-	n = 1
144	282	+	7.0	+	-	-	-	-	n = 0
180	480	+	8.5	+	-	+	-	-	n = 6

Table 2. Southern analysis data of selected clones from the reverse subtraction (tester Y785D2).

Clones	Size (bp)	human placenta	Size (kb)	Y776a5	Y785d2	Y798g12	Y911f5	Y915e4	Similar clones
6	642	+	3.5	-	+	-	-	-	n = 1
8	422	+	4.0	-	+	-	+	-	n = 0
11	-	-	-	+	+	+	+	+	-
17	562	+	9.0	-	+	-	+	-	n = 0
21	-	-	-	+	+	+	+	+	-
24	810	+	5.5	-	+	-	+	-	n = 2
44	164	+	5.5	-	+	-	+	-	n = 0
68	-	-	-	+	+	+	+	+	-
77	583	+	6.0	-	+	+	-	-	n = 0
78	-	-	-	+	+	+	+	+	-
119	-	-	-	+	+	+	+	+	-
132	584	+	10.0	-	+	-	-	-	n = 0
144	389	+	7.0	-	+	-	+	-	n = 1
146	-	-	-	+	+	+	+	+	-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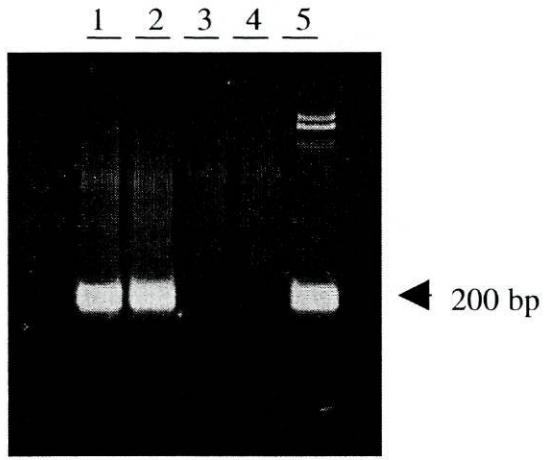
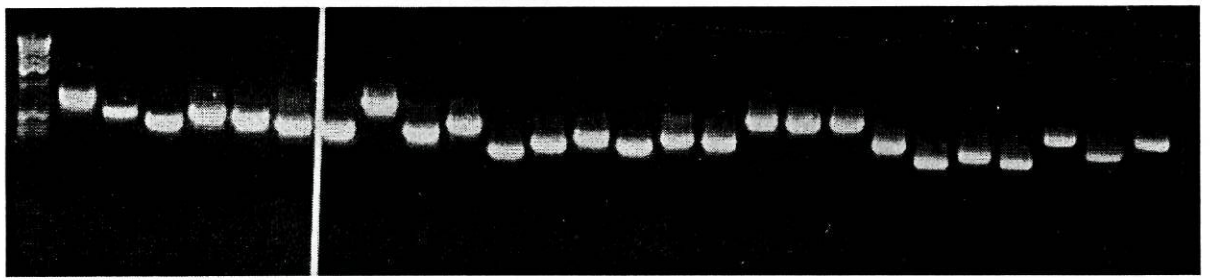
WI3490
WI4792
Y776A5
Y798G12
Y911F5
Y785D2
5 (y776a5)
144 (y776a5)
91 (y776a5)
180 (y776a5)
77 (y785d2)
8 (y785d2)
17 (y785d2)
24 (y785d2)
44 (y785d2)
144 (y785d2)
6 (y785d2)
132 (y785d2)


1 2 3 4 5
200 bp

M cDNA clones



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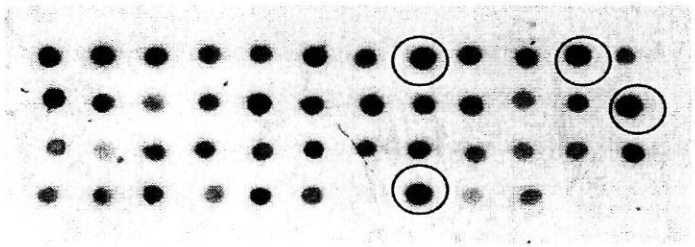

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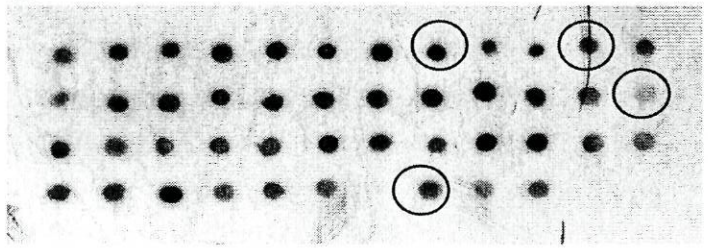

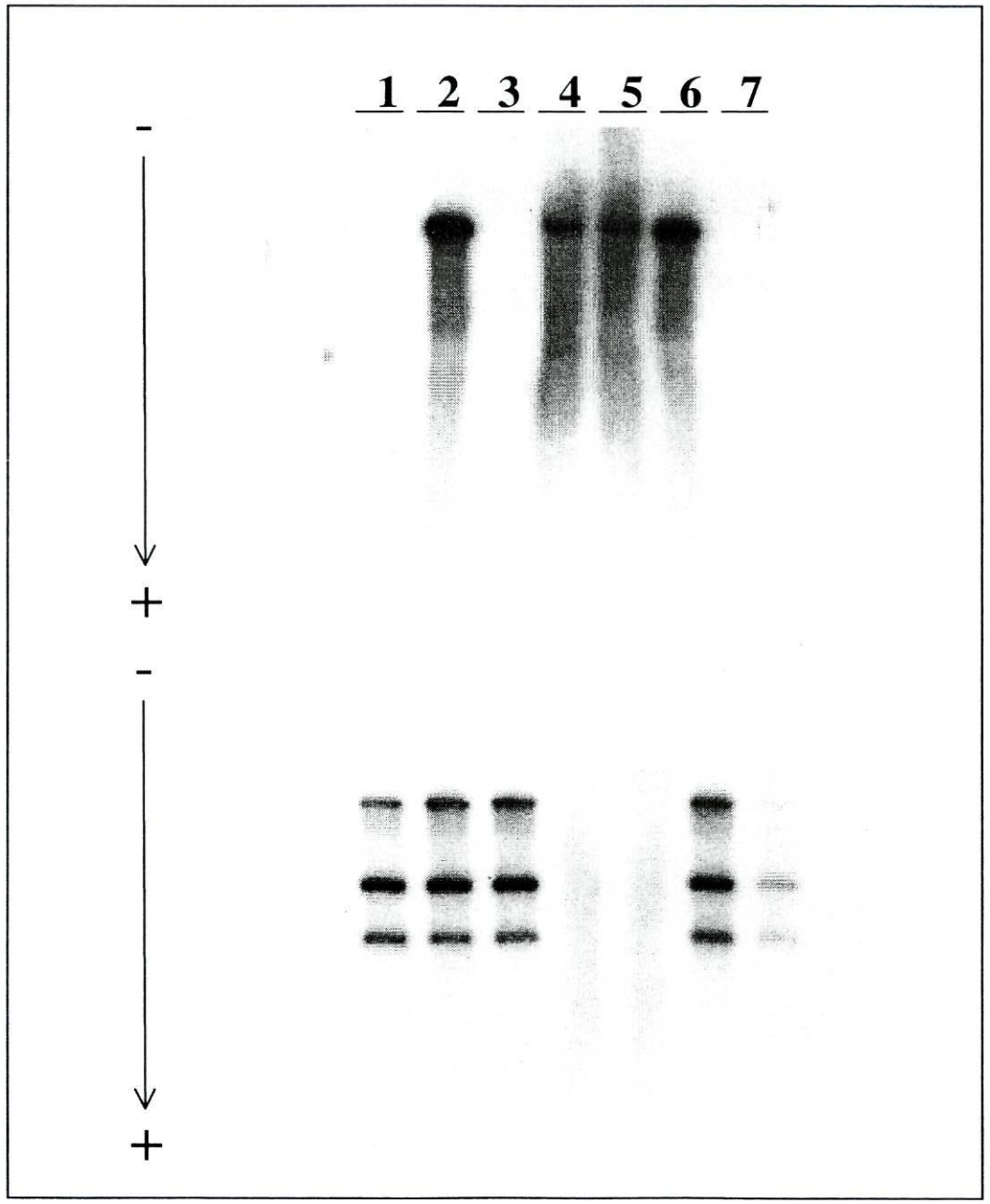
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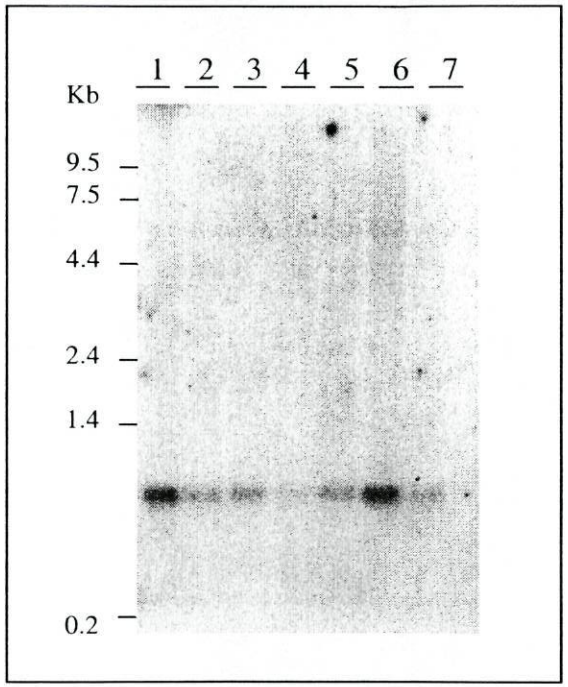
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