FGFR1 and *PLAT* Genes and DNA Amplification at 8p12 in Breast and Ovarian Cancers

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Several chromosomal regions are found to be consistently amplified in human breast cancers. For two of these regions, 8p12 and 10q26, we previously reported the amplification of genes encoding FGF receptors, *FGFR1/FLG* and *FGFR2/BEK*, in about 12% of breast tumors. The *PLAT* gene, encoding the tissue-type plasminogen activator, is also located close to or within the 8p12 region. In the present study, we show that both *FGFR1* and *PLAT* can be amplified in breast as well as ovarian carcinomas. *FGFR1* amplification was detected in 14.5% of breast and 7.8% of ovarian tumors, whereas *PLAT* was found to be amplified in 15.6% and 19.4% of the tumors, respectively. Each gene could be amplified independently of the other. These data raised the question of which gene is selected for amplification at 8p12. In most cases, the levels of expression of *FGFR1* and *PLAT* in breast tumors were comparable to their level of expression in normal mammary tissue. However, *FGFR1* was expressed above the normal level in a certain number of cases. This gene could be a good candidate as "driver" of the 8p12 amplification, but it cannot account for all complex molecular events taking place in this region. *Genes Chrom Cancer 7:219–226 (1993).*

INTRODUCTION

Amplification of a given chromosomal region is a molecular alteration frequently observed in breast and ovarian cancers. The reason for the high incidence in these two tumor types, which account for one quarter of human cancers, has not yet been elucidated. At the molecular level, amplification is thought to begin with the selection for high expression of a gene involved in the initiation and/or the progression of the malignant growth. Such a gene will be referred to here as the "driver" or "target gene" of an amplification unit (amplicon). In breast cancers, the first two oncogenes identified as targets were *MYC* (Escot et al., 1986) and *ERBB2* (Slamon et al., 1987). Their importance as markers of prognosis is still a matter of discussion, but their implication in the cancerous process itself remains unquestioned.

Recently, three other chromosomal regions—8p12, 11q13, and 10q26—have been found to be consistently amplified in breast cancers. Interestingly, these regions contain either *FGF* genes or *FGF* receptor genes (Lammie and Peters, 1991; Gaudray et al., 1992). *FGF3* (*INT2*) and *FGF4* (*HST/K-FGF*) are co-localized in band 11q13 (Nguyen et al., 1988), whereas *FGFR1/FLG* and *FGFR2/BEK* are localized in 8p12 and 10q26, respectively (Ruta et al., 1988; Matteï et al., 1991; Dionne et al., 1992). Moreover, a large proportion of tumors with an amplification of 11q13 also show amplification of *FGFR1*, suggesting the creation of an amplified autocrine loop as a possible mechanism involved in breast carcinogenesis (Adnane et al., 1991; Gaudray et al., 1992). However, the low level or absence of expression of both *FGF3* and *FGF4* in amplified breast tumors, together with the recent identification of the *CNND1* cyclin D gene as the probable driver of 11q13 amplicons (Lammie and Peters, 1991), has cast some doubt on this hypothesis and led us to question the true involvement of *FGFR1* in the formation of the 8p12 amplicon (Gaudray et al., 1992).

Other potential candidate genes in the chromosomal 8p12-q11 and 10q24-qter regions are the tissue-type and urokinase-type plasminogen activators (PA) (Human Gene Mapping 11, 1991). They are thought to play an important role in tumor growth by focally degrading components of the basement membranes, thus favoring the neoangiogenesis, invasiveness, and metastatic spread of a tumor (for reviews see Saksela and Rifkin, 1988; Testa and Quigley, 1990).

Because direct involvement in carcinogenesis through DNA amplification of either the *FGFR* or

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the *PA* gene has never been assessed, we have tested the possibility that the *FLG/FGFR1* or *PLAT* genes are the target genes of the 8p12 amplicon.

MATERIALS AND METHODS

Tumor Samples

Panels of breast tumors (primaries as well as recurrences) were collected during the past 5 yr at the Princess Grace Clinical Center in Monaco, the Antoine Lacassagne Cancer Center in Nice, the Paul Lamarque Cancer Center in Montpellier, the Paoli-Calmettes Institute in Marseille, and the Fondation Bergonié in Bordeaux. Tumors were classified according to the WHO Histological Typing of Breast Tumors (1982).

One hundred three ovarian carcinoma samples were collected at the Paul Lamarque Cancer Center in Montpellier and in the Gynecology and Obstetrics departments of the University Hospitals in Montpellier and Turin. Not all tumors could be typed and staged.

All surgical biopsy specimens were frozen within 30 min of removal and were stored at −80°C before processing.

Probes

FLG/FGFR1 probes were described previously (Adnane et al., 1991; Lafage et al., 1992). The *FGFR1* probe used for dot blot and Northern blot analyses was the 340 bp *Eco*RI-*Bgl*II insert fragment from the pOL10 plasmid. It corresponds to a portion of cDNA coding from the middle of the second immunoglobulin-like loop (IgL) to the middle of the third IgL (amino acids 196 to 313 of the FGFR1 protein; Dionne et al., 1990), recognizing all forms of transcripts. The *PLAT* cDNA probe, a 473 bp *Eco*RI fragment, was derived from plasmid pPAE500. The control probes were: *FGF6* (located at 12p13), 0.35 kb Pv3 (Marics et al., 1989); *MYCN* (located at 2p24), 2.0 kb *Eco*RI (Kohl et al., 1986); *MOS* (located at 8q11), 2.5 kb *Eco*RI (Watson et al., 1982); and *SEA* (located at 11q13), 3.2 kb *Eco*RI (Williams et al., 1988) fragments. The *Gapdh* is derived from a mouse clone (Galland et al., 1990). The β-actin probe was described previously (Moos and Gallwitz, 1983). Probes were labeled by oligolabeling.

DNA Analysis

Genomic DNAs were extracted and analyzed as previously described (Theillet et al., 1989) except when RNA was extracted simultaneously (see below). Two-fold amplifications observed in two different experiments were included.

Dot Blot Analysis

Total RNA was extracted from 150 mg of tumor sample by the acid-guanidinium-phenol-chloroform method (Chomczinski and Sacchi, 1987). Four different amounts of denatured RNA were loaded on Hybond N membranes (Amersham) with a BRL Hybri-dot Manifold apparatus and fixed onto the filter by 5 min UV exposure. Prehybridization and hybridization were performed at 52°C in 50% formamide solution. Membranes were washed four times at room temperature in 2 × SSC, 0.1% SDS for 15 min, then twice at 60°C in 0.1 × SSC, 0.1% SDS for 15 min, and exposed for 5 days. Each filter was then hybridized with a β-actin probe. Hybridization signals were quantified by densitometric scanning (Biocom). For each sample, *FGFR1* RNA levels were expressed as a percentage of the value obtained with a human breast carcinoma cell line 200 resistant to Adriamycin (Adr^{200} MCF-7) (Cowan et al., 1986), which overexpressed *FGFR1*. The β-actin signal was used as internal standard. Mean values were estimated. For each blot, *FGFR1* expression in the MCF-7 parental cell line was also evaluated.

Northern Blot Analysis

Total RNA from frozen tumor tissues or from cell lines was extracted according to Sambrook et al. (1989), with the guanidinium isothiocyanate-cesium chloride method. After centrifugation, the DNA was prepared as described by Zeillinger et al. (1989). Ten micrograms of tumor or cell line RNA were separated on 1% agarose gels containing formaldehyde. RNA was transferred onto Nytran filters (Schleicher and Schuell) and hybridized as described by Stewart and Walker (1989). Hybridization signals were assessed quantitatively by densitometer scanning (LKB).

RESULTS

FGFR1/FLG AND *PLAT* are Frequently Co-amplified in Breast and Ovarian Carcinomas, but Can be Amplified Independently

The amplification status of the *FGFR1* and *PLAT* genes was assessed by Southern blot analysis in panels of 173 breast and 103 ovarian tumors. Internal control probes were the *FGF6*, *MOS*, *MYCN*, and *SEA* genes, which are infrequently altered in these tumors (Adnane et al., 1989; Theillet et al., 1990).

Whereas in breast tumors *FGFR1* and *PLAT*

TABLE 1. Amplification Frequencies of the *FGFR1* and *PLAT* Genes in Human Breast and Ovarian Tumors

Amplifications	Breast tumors	Ovarian tumors
FGFR1	25/173 (14.5%)	8/103 (7.8%)
PLAT	27/173 (15.6%)	20/103 (19.4%)
Co-amplifications	14/173 (8.1%)	6/103 (5.8%)

showed a similar incidence of amplification (14.5 and 15.6%, respectively), in ovarian tumors *PLAT* was amplified twice as frequently as was *FGFR1* (19.4 and 7.8%, respectively) (Table 1). Fourteen of 25 breast tumors and six of eight ovarian tumors amplified for *FGFR1* were amplified for both *PLAT* and *FGFR1* (Table 1). Thus, every possible combination of amplification could be observed: *FGFR1* and *PLAT*, *FGFR1* only, or *PLAT* only (Fig. 1).

We also observed loss of heterozygosity (LOH) in 16 of 90 (17.7%) informative breast tumor samples with the *PLAT* probe, which reveals an *Eco*RI polymorphism (Fig. 1, right panel; compare BT472 DNA to its cognate lymphocyte DNA). LOH was often concomitant with the amplification of the remaining allele (Fig. 1), but could also occur independently of any amplification.

Expression of *FGFR1/FLG* and *PLAT* Genes in Breast Carcinomas

We analyzed the expression of both *FGFR1* and *PLAT* in breast cancers, as well as in samples of normal breast tissue. We determined the modal level of expression of *FGFR1* in breast tumors by dot blot analysis on 64 tumor samples from which only total RNA was available. Levels of expression were estimated with a β-actin probe used as internal control and normalized against that of the

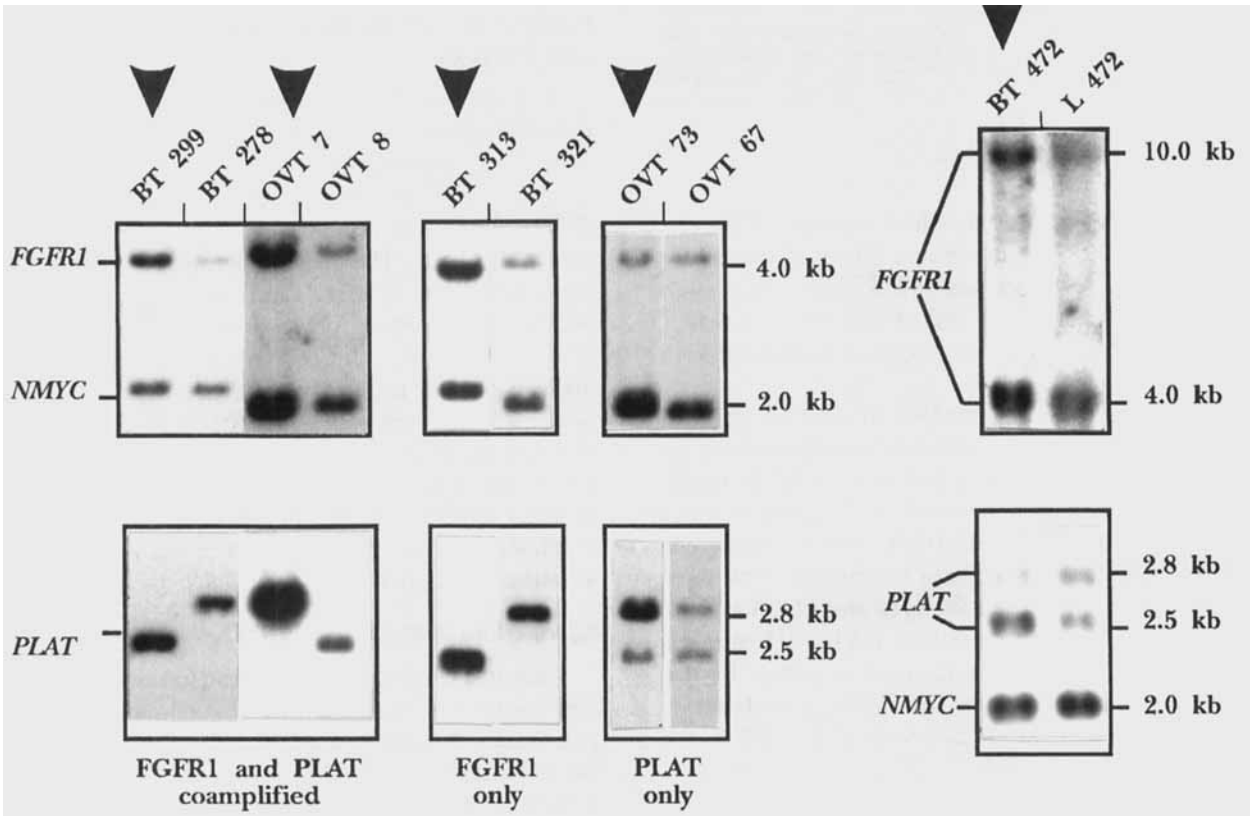

Figure 1. DNA amplification of the *FLG/FGFR1* and *PLAT* genes in breast and ovarian tumors. Representative examples of amplifications observed with various probes are presented. Lanes are identified above with the tumor sample and below with the type of amplification or co-amplification they bear. Amplified tumors are indicated by an arrowhead. A probe for the *MYCN* gene was used for normalization of hybridization levels. Other probes used as controls (not shown here) were *SEA* and *MOS*. With this *FGFR1* probe, two *Eco*RI bands of 6.0 kb and 2.5 kb are usually present; the upper one, giving a weaker signal, has been omitted in the left panel of the figure. The *PLAT* probe detects an *Eco*RI polymorphism (Human Gene Mapping 11, 1991), and the tumors can derive from either a homozygous (for example, OVT 7) or a heterozygous (for example, OVT 73) subject. Because the lanes shown here originate from different Southern blots, there can be differences in the migration rates. The panel at the far right shows an example of a tumor with an amplification of one allele of *PLAT* concomitant with the loss of the other allele.

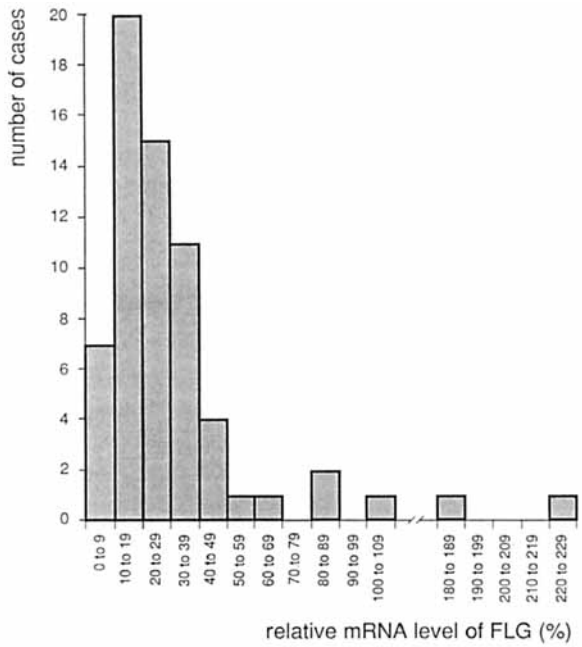

Figure 2. Distribution of relative expression of *FGFR1* mRNA in 64 primary breast carcinomas. The relative amounts of mRNA were quantified as described in Materials and Methods and are expressed in percentage with respect to the Adr^{200} MCF-7 cell line. The range of amounts of *FGFR1* expression is 0 to 221%. The MCF-7 cell line has a relative mRNA level of 17%.

Adr^{200} MCF-7 cell line, which was found to overexpress *FGFR1* (not shown). Most of the breast cancer samples expressed *FGFR1* (Fig. 2) at levels corresponding to 10 to 30% of that found in Adr^{200} MCF-7, but several cases expressed comparable or even higher levels.

Whether the amplification of *FGFR1* contributed to an elevated expression was determined by comparison of RNA expression with DNA amplification. This was performed on 82 tumor samples in which both DNA and RNA could be analyzed. The MDA-MB-134 breast carcinoma cell line, known to be amplified for 8p12 and 11q13 regions (Lafage et al., 1992), and the KATO III stomach carcinoma cell line, known to be amplified for the 10q26 region (Hattori et al., 1990, and data not shown), were used for comparison. *FGFR1* is not amplified in the Adr^{200} MCF-7 cell line (not shown).

Northern blot hybridizations were carried out on these 82 tumor samples, the amplification status of which was analyzed simultaneously. Results are shown in Figure 3 (panel B) and Table 2. *FGFR1* was expressed as a 4.4 kb transcript, and the levels of expression were, in most samples (72/82), comparable to those observed in normal breast tissue (sample 5285). Some tumors (4401, 4420, 4733, 5096, and 5205), however, showed RNA signals comparable to that of the MDA-MB-134. *FGFR1* overexpression often occurred in tumors presenting DNA amplification (Fig. 3A: 4420, and 4733), but overexpressing tumors were not always amplified (4401, 5096) and some amplified tumors (4700, 4885) did not overexpress *FGFR1*. In total, *FGFR1* RNA overexpression was found in ten of 82 breast tumors and was correlated statistically with DNA amplification (Table 2). However, we did not observe a perfect overlap of both events.

In comparison, the levels of *PLAT* expression were relatively uniform. With the exception of some tumors (4401, 4414 in Fig. 4), *PLAT* was not overexpressed. Elevated RNA levels did not correlate with amplification of the gene, and, conversely, amplified tumors did not overexpress *PLAT* RNA. Thus, this gene is clearly excluded as an active player in the formation of the 8p12 amplicon.

Frequent Co-amplification of 8p12 and 11q13 Regions

Amplification in other chromosomal regions was determined for most of the breast tumor samples. A striking association between amplification at 8p12 and at 11q13 was noted. We observed that 31% of the tumors amplified at 8p12 were also amplified at 11q13. The two events were statistically correlated ($P = 0.003$). The concordance of amplification was most obvious when *FGFR1* and *CCND1* (*PRAD1*, *CYCD1*) were considered as markers of their respective amplicons. Indeed, ten of 25 (40%) tumors with an amplified *FGFR1* gene also had an amplified *CCND1* locus (Fig. 5). This association was even more noticeable in the tumors in which *FGFR1*, and not *PLAT*, was the only 8p12 amplified marker. It was not observed with other amplifications, such as that of *ERBB2* (not shown).

Correlations With Clinico-pathological Parameters

Statistical analysis of the correlations between DNA amplification at 8p12 and clinico-pathological parameters was performed on 173 breast tumors. No statistically significant correlation could be observed. However, a trend of preferential association between amplification and estrogen-positive tumors was noted. This is in agreement with the trend observed previously on an independent series of tumors analyzed for *FGFR1* amplification only (Adnane et al., 1991). Interestingly, although amplification and overexpression of *FGFR1* did not strictly overlap, a similar trend of association was

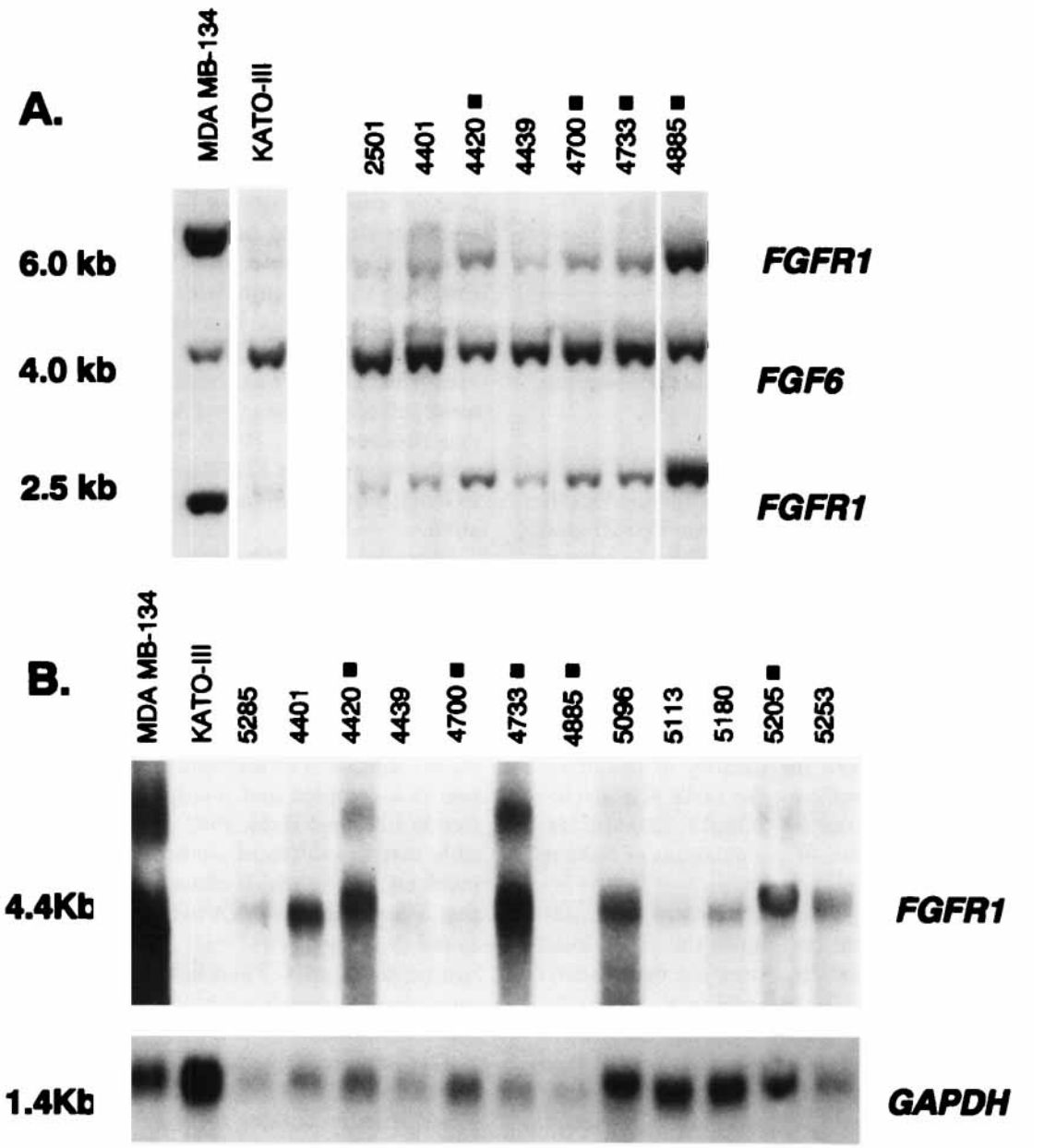

Figure 3. Amplification and expression of *FGFR1* and *PLAT* genes in breast carcinomas. **A:** Selected tumor sample DNAs were hybridized to *FGFR1*. Here, *FGF6* was used as control probe. Southern blot hybridizations of DNAs from the mammary carcinoma cell line MDA-MB-134 and from the stomach carcinoma cell line KATO III are shown for comparison. **B:** Ten micrograms of total RNA extracted from breast carcinoma samples and the two tumor cell lines were analyzed by Northern blot hybridization (see Materials and Methods) with an *FGFR1* cDNA probe. A transcript of 4.4 kb was observed in most samples. Overexpression of *FGFR1* was evidenced in the mammary carcinoma cell line MDA-MB-134, which was used as control for overexpression. No 4.4 kb signal was detected in the stomach cancer cell line Kato III, which has an amplified and overexpressed *BEK/FGFR2* gene (Hattori et al., 1990, and data not shown), and which was used as a control for the absence of cross-reactivity between *FGFR1* and *FGFR2*. A faint band of 3.5 kb corresponding to the *BEK* transcript (Hattori et al., 1990) could be observed on some long exposures. The *Gapdh* probe was used as control probe. Black squares indicate that the 8p12 region is amplified in the tumor.

observed with the 146 tumors analyzed for expression; i.e., overexpressed tumors were found frequently among the small, low-grade, estrogen-positive tumors.

DISCUSSION

Several conclusions can be drawn from these results. 1) Amplification of the 8p12 chromosomal region occurs not only in breast but also in ovarian

TABLE 2. Comparative Status of *FGFR1* Gene Amplification and Overexpression

RNA expression[a]	Normal copy number[a]	Amplified[a]	Total
Normal level	70	2	72
Overexpression	4	6	10
		$P = 0.00015$[b]	
Total	74	8	82

[a]Levels of amplification and RNA expression were estimated as described in Materials and Methods.
[b]Statistical correlations (Chi^2 and Fisher exact tests) were calculated on an 80386-compatible computer with Epiinfo version 5.00 software from CDC, Atlanta, GA.

tumors; 2) *PLAT* can be amplified independently of *FGFR1* and vice versa; 3) both *FGFR1* and *PLAT* are expressed in breast tumors; 4) overexpression and DNA amplification of *FGFR1* and *PLAT* do not overlap strictly; 5) co-amplification of genes from 8p12 and 11q13 regions occurs frequently; and 6) tumors amplified for 8p12 tend to be small, well-differentiated tumors.

Three aspects of our findings need further discussion. They concern the identity of the driver gene of the 8p12 amplicon, the molecular mechanism that sustains the 8p12-11q13 co-amplification, and the relevance of the deletions of 8p12 to the cancerous process.

We show that, in breast and ovarian cancer, amplification at 8p12 can encompass the *PLAT* locus in addition to *FGFR1*. However, the two markers were not systematically co-amplified. Moreover, the patterns of amplification were not identical in breast and in ovarian tumors. This finding led us to question the identity of the driver gene of the 8p12 amplification process. The hypothesis of a tyrosine kinase gene whose amplification would be selected for in DNA amplifications in human breast and ovarian cancers seemed attractive. Similarly, the plasminogen activators could also appear as good candidates. A body of evidence supports the involvement of PAs, especially of *PLAU,* in breast cancer progression (O'Grady et al., 1985; Sappino et al., 1987; Jänicke et al., 1989; Duffy et al., 1990; Foucré et al., 1990). Therefore, RNA expression studies were needed for determination of which gene was actively involved.

Whereas expression analysis clearly excludes an active role of *PLAT,* no definitive conclusion on *FGFR1* can be drawn. Some tumors amplified for *FGFR1* expressed low levels of RNA, but the majority (6/8) of the amplified tumors, and one amplified cell line, showed high levels of expression. These data are strong arguments in favor of the involvement of *FGFR1*. Recent reports have documented the expression of *FGFR* genes in breast carcinomas (Ding et al., 1992; Luqmani et al., 1992). Unfortunately, the status of *FGFR1* amplification was not analyzed in these studies. FGF receptors are indeed frequently detected on breast tumor cells (Peyrat et al., 1992) and it is known that FGF1/bFGF stimulates mammary cells (Takahashi et al., 1989). In a more general way, elevated transcription of *FGFR1* could extend beyond breast tumors, for it has been observed in glioblastoma cell lines (Saxena and Ali, 1992) and melanomas (Becker et al., 1992). Taken together, these results suggest that FGFRs, and more specifically FGFR1, may contribute to malignant cell proliferation.

However, *FGFR1* cannot account for all amplification events recorded at 8p12, especially when *PLAT* is the only amplified marker. Furthermore, although this case should not be overstated, the observation made on tumor 4885, in which *FGFR1* is clearly amplified, but not overexpressed, may also be representative of an alternative mechanism for the formation of the 8p12 amplicon. Amplification is a complex and poorly understood process (see Windle and Wahl, 1992, for review). It is possible that two different molecular mechanisms are involved, one in which elevated *FGFR1* transcription is coupled with DNA amplification, in either a causal or opportunistic way, and one in which the two are dissociated. Therefore, a greater number of cases should be analyzed before any definitive conclusion is drawn.

The important overlap of 8p12 and 11q13, and particularly of *FGFR1* and *CCND1* amplifications, is sufficiently striking to raise some questions about a possible mechanism related to the genesis of breast tumors. One possible explanation is that the activation of a pair of genes acting synergistically is induced or potentiated by this co-amplification. Theoretically, the co-amplification may be a double amplification of two independent regions, or a true co-amplification within the same unit. We have indeed observed that the MDA-MB-134 cell line presented such a co-amplification of the 8p12 and 11q13 regions linked de novo (Lafage et al., 1992). Whether the co-amplification found in the tumors relies upon physical association will have to be determined.

The frequency of deletions observed at 8p12 requires further investigation of the extent and actual

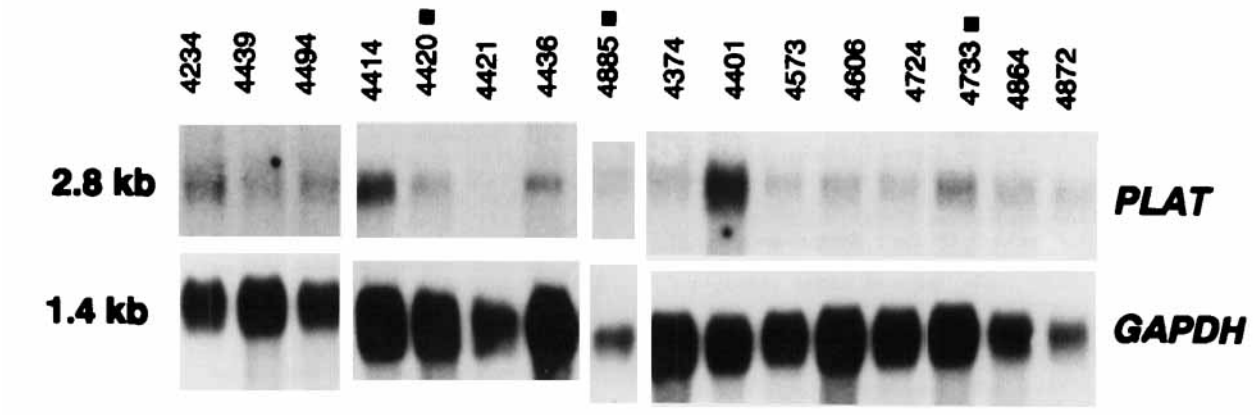

Figure 4. Northern blot analysis of *PLAT* expression in breast carcinomas. The *PLAT* probe was hybridized to 10 μg of breast tumor total RNA. Black squares indicate that the 8p12 region is amplified in the tumor.

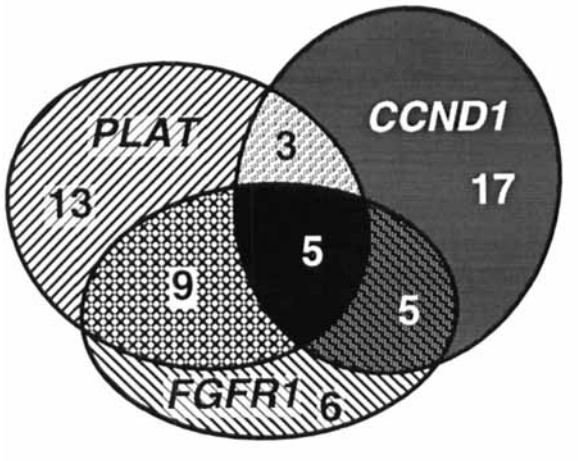

Figure 5. Schematic representation of the incidence of 8p12-11q13 co-amplifications. The number of amplified or co-amplified tumors in each sub-population is indicated.

incidence of the chromosomal losses. One possibility is that the deleted material includes the locus for an anti-oncogene. Such a locus, located between *PLAT* and 8pter, has been hypothesized recently to play a role in several carcinomas (Bergerheim et al., 1991; Worsham et al., 1991; Emi et al., 1992; Van der Bosch et al., 1992). The chromosomal breakage that initiates the loss of this locus could be followed, on a chromosome, by amplification events. The chromosome breakage model has been suggested to explain amplification processes (Windle and Wahl, 1992).

The main conclusion drawn from our data is that, although *FGFR1* remains a good candidate as the gene under selection in the 8p12 amplicons, amplification events taking place in this region may involve at least one additional, as yet unidentified, genetic element. Identification and characterization of this gene will constitute an important step toward the elucidation of mechanisms involved in carcinogenesis.

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