Ras-related TC21 is activated by mutation in a breast cancer cell line, but infrequently in breast carcinomas in vivo

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Summary Activating ras mutations are found in many types of human tumour. Mutations in Harvey (H-). Kirsten (K-) and neuronal (N-) ras are, however, rarely found in breast carcinomas. TC21 is a ras family member that shares close homology to H-. K- and N-ras. and activating mutations have been found in ovarian carcinoma and leiomyosarcoma cell lines. We have examined panels of cDNAs from breast, ovarian and cervical cell lines, and primary and metastatic breast tumours for mutations in TC21 using a single-strand conformational polymorphism (SSCP)-based assay. One breast cancer cell line. CAL51, exhibited an altered SSCP pattern, compared with normal tissue, which was due to an A-T base change in codon 72, causing a predicted Gln-Leu activating mutation. Of nine primary and 15 metastatic breast tumour cDNAs analysed, none exhibited an altered pattern by SSCP. The apparently wild-type pattern by SSCP analysis was confirmed by sequence analysis of some of the cDNAs assayed. Thus. we conclude that mutations in TC21 are uncommon in breast carcinomas.

Keywords: breast cancer. ras gene. TC21: single-strand conformational polymorphism

Mammalian ras genes encode a family of small GTPases involved in signal transduction pathways leading to cell growth and differentiation. RAS proteins are inactive in the GDP-bound state and are activated in response to receptor protein tyrosine kinases or other stimuli to become GTP bound (Barbacid. 1987). Guanine nucleotide exchange factors (GEFs) positively regulate RAS by promoting the active GTP-bound form; and GTPase-activating proteins (GAPs) negatively regulate RAS by accelerating the GTPase activity of RAS (reviewed in Boguski and McCormick. 1991). Active RAS interacts with effectors to transduce its signals, the most well-characterized interaction being that with RAF-1. Active RAS binds to RAF-1 and recruits it to the plasma membrane, where it activates the MAP kinase pathway. This leads to several downstream events, including control of gene transcription (reviewed by Marais and Marshall. 1996).

Mutated ras genes were first discovered because of their ability to transform NIH/3T3 cells after DNA transfection. Subsequently, many human tumours were shown to harbour ras point mutations, most commonly in codons 12. 13 and 61 (Bos. 1989). Alterations in these amino acids lead to a decreased rate of GTP hydrolysis or a failure to respond to GAP, thus resulting in an accumulation of active GTP-bound RAS. Harvey (H-). Kirsten (K-) and neuronal (N-)ras were the first to be identified, and are the most well-characterized with respect to mutational status in tumours. There is a large variation in the frequency of mutations between tumour types. The highest incidence thus found is in pancreatic tumours, where over 80% have mutated K-ras genes, whereas mutations are rarely seen in either H-. K- or N-ras in breast tumours (Bos. 1989: Clark and Der. 1995). Ras mutations have been described in some mucinous ovarian cancers (Ichikawa et al. 1994: Cuatrecasas et al. 1997), but generally appear to be a rare event in ovarian carcinomas (van’t Veer et al. 1988: Berchuck and Carney. 1997). The incidence in cervical cancer is not as clear: some reports describe a high frequency of ras mutations (Riou et al. 1988: Wong et al. 1995), others have found a very low frequency (Bos. 1988: Willis. 1993).

One possible explanation for the absence of ras mutations in breast cancer could be that deregulation of RAS function does not lead to uncontrolled growth of breast epithelial cells. Human phaeochromocytomas do not have mutations in ras, and the introduction of oncogenic RAS into PC12 cells, which are rat phaeochromocytoma cells, leads to differentiation and growth arrest (Bar-Sagi and Feramisco. 1985: Noda et al. 1985). This does not appear to be the case for breast epithelial cells. For example, expression of oncogenic H-ras leads to the transformation of the breast epithelial cell line MCF 10A (Basolo et al. 1991). Elevated levels of H-ras product have been detected in human breast tumours (Watson et al. 1990). Another possibility is that deregulation of RAS pathways via other mechanisms not involving mutations in ras genes may be important. Alternatively, other members of the ras family may play more major roles in growth control in breast cancer, and mutations may be found in these genes.

TC21 is a ras family member cloned from a human teratocarcinoma cell line. It shows overall 60% nucleotide and 55% amino acid identity with H-. K- and N-ras, with a strictly conserved effector domain (Drivas et al. 1990). This is the closest homology to H-. K- and N-ras genes of any of the ras superfamily cloned to date. There are several pieces of evidence to suggest that TC21 may play a role in human cancer. Codons 22. 23 and 72 in TC21 are analogous to those whose in vivo mutation leads to oncogenic activation in H-. K- and N-ras (Graham et al. 1994). When codons 22 and 72 were experimentally mutated in TC21, 22 Glu to Val, and 72 Gln to Leu, the result was a transforming activity equivalent to that of oncogenic ras (Graham et al. 1994). A form of the 72 Gln to Leu mutation in TC21 has been cloned from an ovarian carcinoma cell
line (Chan et al. 1994), and the transforming capacity and tumorigenicity of this mutation in NIH/3T3 cells was confirmed. An insertional mutation of 9 bp at codon 24 in TC21 has been identified in a human leiomyosarcoma cell line, and this displays high transforming activity in NIH/3T3 cells (Huang et al. 1995).

Whereas most experiments on transformation by mutant TC21 have been performed in murine fibroblast cells, mutants 22 Glu to Val and 72 Glu to Leu have been shown to transform MCF 10A, spontaneously immortalized human mammary epithelial cells (Soule et al. 1990), causing altered cell morphology and allowing colony formation in soft agar (Clark et al. 1996). It is therefore possible that aberrant TC21 signalling may be involved in breast tumour progression. To examine the possibility that TC21 may be mutated in breast cancer, we have developed a single-strand conformational polymorphism (SSCP)-based assay to look for equivalent mutations in breast cancer cell lines and primary and metastatic breast carcinomas. In view of the low incidence of K- and H-ras mutations reported in ovarian tumours, and the identification of a TC21-activating mutation in an ovarian cancer cell line, A2780, we surveyed a panel of ovarian cancer cell lines. ras mutations may play an important role in the progression of cervical cancer, but the reported frequency of mutation varies between studies. As alternative ras gene family members may be mutated in those not harbouring K- or H-ras mutations, we examined a panel of cervical carcinomas and cell lines for activating TC21 mutations.

**MATERIALS AND METHODS**

### SSCP

RNA was isolated using the RNAagents® Total RNA Isolation System (Promega), by a modification of the method of Wilkinson (1988) or using guanidinium isothiocyanate (Chirgwin et al. 1979). cDNA was prepared as described previously (Barker et al. 1995). Oligonucleotides primers 5'TACCGGCCTCGGTGTTGGTCGG3' (sense) and 5'TATCTGGACTGAAAAGACC3' (antisense) were designed to amplify a 249-bp region of TC21 from nucleotides 40 to 289, spanning all three potential activating mutation sites.

Amplifications were carried out using cDNA derived from 0.2 μg of original RNA in 60 mm KCl, 15 mm Tris-HCl pH 8.8, 1.75 mm MgCl2, 200 μM of each dNTP, 3 μCi [α-32P]dCTP, 20 pmol of each primer and 1 unit of Taq polymerase in 25 μl. Each reaction was cycled 30 times at 94°C for 1 min, 55°C for 2 min and 72°C for 1 min. An aliquot of 4 μl of each reaction was mixed with 4 μl of SSCP gel loading buffer (95% deionized formamide, 0.025 M EDTA pH 8.0, 0.005% bromophenol blue, 0.005% xylene cyanol), heated at 95°C for 5 min, and chilled on ice for 1 min. Underneath samples were kept on ice. An aliquot of 4 μl of either denatured or undenatured samples was loaded onto 6% acrylamide, 1×TBE, 10% glycerol gels. Fragments were resolved by electrophoresis at 5 W for 15 h: gels were dried and exposed to X-ray film at -70°C overnight.

### Subcloning and sequencing

PCR reactions were carried out as for SSCP, but without [32P]dCTP. Amplified products were resolved on 1.5% agarose TAE gels, excised and purified using a QIAEX II Gel Extraction Kit (Qiagen). Purified products were subcloned into the pGEM®-T Easy vector (Promega) according to the manufacturer’s instructions. Sequences were determined by the dideoxy method of Sanger et al (1977) using a Sequenase Version 2.0 kit (United States Biochemicals, USB).

### Direct PCR sequencing

PCR amplifications were carried out either as above for subcloning, or with cloned Pvu DNA polymerase (Stratagene), using the manufacturer’s buffer and 200 μM of each dNTP. Amplified products were purified using a QIAEX II Gel Extraction Kit. Sequence reactions using a Sequenase Version 2.0 Kit (USB) were performed on approximately 30 ng of purified product and an excess of primer (1 μg 10 μl⁻¹).

### RESULTS

**SSCP analysis of breast cancer cell lines**

The genomic structure of TC21 has not yet been reported, therefore mutation analysis was performed on cDNA. Primers that bracket the region containing all three potential activating mutation sites were used to amplify a single band of the predicted size by PCR. SSCP gel running conditions that detect an altered mobility caused by a single base pair change were assessed using cDNA from normal mammary tissue, and from the ovarian cell line A2780, which has an A-T transversion in codon 72 leading to
a Gln to Leu mutation (Chan et al. 1994) (Figure 1A). Two bands representing two complementary strands were observed, and there was a shift in mobility of the higher band in the A2780-derived PCR product compared with the normal tissue as expected (Huang et al. 1995). Ten breast carcinoma cell lines were examined (Figure 1B). The cell line MDA MB 415 failed to amplify a product on two independent determinations. MDA MB 157.231, 468 (not shown), SKBr3, BT474, MCF 7, ZR75.1 and T-47D all exhibited a wild-type pattern on SSCP analysis. SSCP analysis of cDNA from MCF 10A cells revealed a wild-type pattern of bands, consistent with the observations that these cells when transfected with mutant TC21 exhibit altered morphology and enhanced growth in soft agar. One cell line, CAL51 (Gioanni et al. 1990), revealed a shift in mobility of the upper band characteristic of the pattern seen with A2780 cells. The bands marked with arrows in Figure 1B represent the two complementary strands. Extra bands are sometimes seen (marked with an asterisk); these are irreproducible and are not indicative of sequence changes.

**TC21 is mutated in CAL51 cells**

To investigate the cause of the shift in mobility seen in the SSCP analysis of CAL51 cell line cDNA, the sequence of the derived PCR product was determined. An A to T base change in codon 72, causing a predicted Gln→Leu mutation identical to that seen in the cell line A2780, was seen. Figure 2 shows the sequence of CAL51 in this region compared with the wild-type TC21 sequence from normal tissue. SSCP analysis of A2780 failed to detect a wild-type allele: this is consistent with the observations of Huang et al. (1995). A wild-type allele was not observed in the SSCP analysis of CAL51 cells also, and sequencing revealed the mutated sequence at this codon. Sequencing of the PCR products of other breast cancer cell lines confirmed the SSCP results, in that only wild-type sequences were present (data not shown).

**SSCP fails to detect TC21 mutations in ovarian or cervical cancer cell lines**

Twelve ovarian and eight cervical cancer cell lines were examined for TC21 mutation by SSCP. Amplified products were detected using cDNA from eight of the ovarian and seven of the cervical cell lines, and in all cases the SSCP analysis gave a wild-type pattern. When two of the cell line PCR products were sequenced, only wild-type sequences were observed.

**Lack of TC21 mutation in a panel of primary and metastatic breast tumours**

Five normal mammary tissue-derived cDNAs were subjected to SSCP analysis and all gave wild-type TC21 patterns. Each of nine primary breast cancers that gave an amplified TC21 product gave a wild-type pattern. Of 15 metastatic breast tumours from lymph nodes, 13 produced a wild-type TC21 pattern. Figure 3 shows a representative sample, whereas one product produced an apparently altered pattern. This cDNA and one obviously normal cDNA were amplified by PCR and sequenced. Sequencing of the product from one node revealed some silent changes with respect to the published cDNA sequence, but none resulting in amino acid changes.

**DISCUSSION**

The low frequency of H- ras, K- and N-ras mutations in breast tumours has led us to undertake this study to examine the possibility that the ras superfamily member TC21 is mutated in breast cancers. None of the nine primary or 15 metastatic breast carcinoma samples that we analysed were mutant for TC21. We conclude that TC21 mutations are an infrequent event in the development of breast tumours. Of the panel of breast, ovarian and cervical cancer cell lines analysed, only one. the breast carcinoma cell line CAL51, had a mutation in TC21.

CAL51 cells were isolated from a malignant pleural effusion of a woman with a metastatic breast adenocarcinoma. The cells exhibit morphological, structural and immunohistochemical characteristics of mammary epithelial cells. They will form colonies in soft agar and are tumorigenic in nude mice (Gioanni et al. 1990). Unlike the majority of established breast carcinoma cell lines they have an apparently normal karyotype (Gioanni et al. 1990; S Birdall. personal communication). The genetic alterations responsible for the neoplastic phenotype of CAL51 are not known (they lack mutations in the p53 gene. Theile et al. 1994). To our knowledge, this
mutation in TC21 is the only genetic aberration reported for CAL51 cells. When this mutant form (72, Glu-Leu) was transfected into MCF10A cells, it conferred the ability to form colonies in soft agar, but not tumorigenicity in nude mice (Clark et al. 1996). It is possible that the ability of CAL51 cells to form colonies in soft agar is at least partially due to this mutation in TC21.

Mutations in ras genes do not appear to be a common event in breast, cervical or ovarian tumours (with the possible exception of mucinous ovarian tumours, see Introduction). Although it could be hypothesized that cell type-specific differences in the likelihood of acquiring co-operating mutations account for the apparent tumour type bias of ras mutations, the deregulation of RAS pathways in the development of tumours such as those of the breast should not be discounted. Activated RAS is able to transform breast epithelial cells (Basolo et al. 1991), and chemically induced rat mammary tumours harbour ras mutations (Sukumar, 1990). Human breast epithelial cells transformed by carcinogens acquire activating ras mutations (Zhang et al. 1994). In addition, several studies in transgenic mice have found that activated and/or overexpressed ras alleles can predispose to the development of mammary tumours (Andres et al. 1987; Sinn et al. 1987; Mangues et al. 1992). In view of the above findings, there is a possibility that other components of RAS signalling pathways are altered in breast, cervical and ovarian tumours, thus having the equivalent effect without an activating ras mutation. Oncogenic RAS is less sensitive to GAPS leading to an accumulation of active GTP-bound RAS: a reduction in GAP activity could have the same effect as mutated RAS. The two best characterized mammalian RAS GAPS are p120 GAP and NF1 GAP. Neurofibromatosis type 1 (NF1) is an autosomal dominant condition that predisposes to certain types of carcinoma. There is evidence to suggest that NF1 may function as a tumour suppressor (Legius et al. 1993): this may be due to decreased RAS-GAP activity. Tumours and cell lines derived from tumours of NF1 patients have decreased NF1 GAP activity and increased levels of RAS-GTP (Basu et al. 1992; DeClue et al. 1992; Bollag et al. 1996; Guha et al. 1996). NF1 mutations have also been found to occur in spontaneously arising tumours (Li et al. 1992; Andersen et al. 1993). However, some cell lines established from tumours occurring in non-NF1 patients have reduced levels of NF1 but not elevated RAS-GTP levels (Johnson et al. 1993). It may be that loss of NF1 function(s) independent from GAP activity play a role in the phenotype of these cells. It is hypothesized that GAPS may function as downstream effectors of RAS in addition to their regulatory activity (Clark and Der, 1995). There have been cases of breast cancers of an aggressive nature reported in NF1 patients (Teh et al. 1997). It may therefore prove informative to make a more detailed analysis of NF1 or p120 GAP expression and sequence in breast and other tumours.

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