

Analysis of the TGF β functional pathway in epithelial ovarian carcinoma

KM Francis-Thickpenny¹, DM Richardson^{1,4}, CC van Ee¹, DR Love², IM Winship³, BC Baguley⁴, G Chenevix-Trench⁵ and AN Shelling¹

¹Research Centre in Reproductive Medicine, Department of Obstetrics and Gynaecology, National Women's Hospital, Auckland; ²School of Biological Sciences, University of Auckland, Auckland; ³Department of Molecular Medicine, University of Auckland, Auckland; ⁴Auckland Cancer Society Research Laboratories, University of Auckland, Auckland, New Zealand; and ⁵The Queensland Institute of Medical Research, c/o Royal Brisbane Hospital Post Office, Herston, QLD 4029, Australia

Summary Epithelial ovarian carcinoma is often diagnosed at an advanced stage of disease and is the leading cause of death from gynaecological neoplasia. The genetic changes that occur during the development of this carcinoma are poorly understood. It has been proposed that IGFIIR, TGF β 1 and TGF β RII act as a functional unit in the TGF β growth inhibitory pathway, and that somatic loss-of-function mutations in any one of these genes could lead to disruption of the pathway and subsequent loss of cell cycle control. We have examined these 3 genes in 25 epithelial ovarian carcinomas using single-stranded conformational polymorphism analysis and DNA sequence analysis. A total of 3 somatic missense mutations were found in the TGF β RII gene, but none in IGFR1 or TGF β 1. An association was found between TGF β RII mutations and histology, with 2 out of 3 clear cell carcinomas having TGF β RII mutations. This data supports other evidence from mutational analysis of the PTEN and β -catenin genes that there are distinct developmental pathways responsible for the progression of different epithelial ovarian cancer histologic subtypes. © 2001 Cancer Research Campaign <http://www.bjcancer.com>

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Ovarian cancer is often diagnosed at advanced stages of the disease. Despite improvements in chemotherapy and surgical treatment, the overall 5-year survival rate for patients with epithelial ovarian cancer remains at only 35% (Landis et al, 1998). Overall, the molecular changes that underlie the initiation and development of this tumour are poorly understood. A greater understanding of these complex molecular events could lead to improvements in screening techniques and may ultimately provide new targets for chemotherapeutic drugs and gene therapy.

The transforming growth factor- β (TGF β) family is a group of structurally related polypeptides with diverse functions. There are at least 3 highly homologous TGF β isoforms in humans: TGF β 1, TGF β 2 and TGF β 3. TGF β 1 is the best-characterized TGF β isoform, and is known to regulate cell proliferation and differentiation, and can inhibit or stimulate cell growth in different cell types (Yan et al, 1994). TGF β 1 is also known to have an important role in extracellular matrix deposition, cell adhesion, angiogenesis, and immune function (Polyak, 1996). In most cell types, TGF β is a potent inhibitor of cell growth, arresting cell cycle progression in late G₁ phase. This growth suppression is associated with the increased expression or activities of several cyclin-dependent kinase inhibitors, including p15, p21 and p27 (Rich et al, 1999). Critical mutations in the TGF β pathway should also lead to the loss of the ability of the cell to undergo apoptosis. TGF β is therefore a putative tumour suppressor gene and diminished

responsiveness to TGF β is a common feature of epithelial cancers.

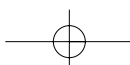
TGF β 1 is secreted from most cells in its latent form, by the non-covalent association with latency-associated protein (LAP). LAP is a homodimer formed from the pro-peptide region of TGF β 1, and is in turn linked to the latent TGF β -binding protein (LTBP). Latent-TGF β 1 must undergo cleavage from this complex in order to become activated. LTBP targets latent TGF β 1 to the extracellular matrix, where it interacts with the insulin-like growth factor II receptor (IGFIIR), itself a putative tumour suppressor gene (Munger et al, 1997). It is thought that IGFIIR orientates latent-TGF β 1 for proteolytic cleavage. Once activated, homodimeric TGF β 1 binds to the constitutively active TGF β receptor type II (TGF β RII). This binding is necessary for recruitment and activation of TGF β receptor type I (TGF β RI). The TGF β RII phosphorylates specific serine residues immediately upstream of the serine-threonine kinase of TGF β RI, resulting in its activation. An anchor protein called Smad anchor for receptor activation (SARA) then recruits Smad2 and Smad3 to the activated TGF β RI (Tsukazaki et al, 1998). Smad2 and Smad3 become transiently associated with, and phosphorylated by, the activated TGF β RI. This association results in the formation of a complex with Smad4. This Smad complex migrates to the nucleus, and recruits other transcription factors and stimulates the expression of specific target genes. The escape from its role in growth inhibition may allow TGF β to act in tumour cells to promote tumourigenicity through its other functions, such as by the stimulation of angiogenesis, extracellular matrix deposition and immunosuppression. Therefore defects in the TGF β pathway might be expected to be associated with cancer development, and mutations in the coding sequence of mature TGF β 1 have been reported in ovarian (Cardillo et al, 1997a), breast (Cardillo et al, 1997b), and colon

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Correspondence to: AN Shelling



cancer (Cardillo and Yap, 1997).

IGFIIR has been proposed as a putative tumour suppressor gene due to its ability to bind and degrade the mitogen IGF-II, promote activation of the growth inhibitor TGFβ, and regulate the targeting of lysosomal enzymes (Jirtle et al, 1994). Loss of heterozygosity (LOH) at the IGFRII locus at 6q26-27 is common in hepatocellular carcinomas and point mutations occur in the remaining IGFIIR allele in 25% of hepatocellular carcinomas with LOH, supporting the role of IGFIIR as a tumour suppressor gene (de Souza et al, 1995a, 1995b). LOH at the IGFIIR locus is also a common occurrence in ovarian cancer (Cooke et al, 1996), and appears to be an early event in tumorigenesis development (Chenevix-Trench et al, 1997), but mutations in this gene have not been reported in epithelial ovarian carcinoma.

Both TGFβRII and TGFβRI are transmembrane serine/threonine kinase receptors, and epithelial cells lacking them are completely unresponsive to the inhibitory response of TGFβ1 (Chen and Derynck, 1994). Several studies have shown that insensitivity to the growth inhibitory effects of TGFβ are most commonly due to alterations of the TGFβRII gene. Loss of expression of TGFβRII protein is a frequent event in prostate cancer and reintroduction of wild-type TGFβRII into prostate cancer cells lacking TGFβRII restores the growth inhibitory response by exogenous TGFβ1 (Gerdes et al, 1998; Guo and Kyprianou, 1998). In their study of ovarian cancer, Lynch et al (1998) found mutations in 6 of 24 samples (25%) in TGFβRII. These mutations included 4 missense mutations within the highly conserved serine/threonine kinase domain, 1 missense mutation within the transmembrane domain, and a single frameshift mutation in the poly(A) microsatellite region. Mutations in TGFβRII have also been reported in squamous carcinoma of the head and neck (Garrigue-Antar et al, 1995; Wang et al, 1997).

A recent report by Wang et al (2000) looked at components of the TGFβ signal transduction pathway, and screened for mutations in TGFβRI, TGFβRII, Smad2 and Smad4. Unlike Lynch et al (1998), they did not find exonic mutations in TGFβRII. However, a frameshift mutation in exon 5 of TGFβRI in 10 of 32 tumour samples (31.3%) was identified. This mutation was associated with the absence of TGFβRI expression.

It has been proposed that IGFIIR, TGFβ1, and TGFβRII

function as a unit, and that somatic loss of function mutations in any 1 subunit could lead to disruption of the TGFβ1 growth inhibitory pathway and subsequent loss of cell cycle control. We screened for mutations in these 3 genes in 25 epithelial carcinomas. If mutations to these genes were identified, this would suggest that the TGFβ1 inhibitory pathway is important in the development of epithelial ovarian cancer and if these mutations are mutually exclusive in individual cancers, it would support the hypothesis that these genes act as a functional unit (Haber and Fearon, 1998).

METHODS

Patient DNA samples

Tumour samples from 25 women with various stages of ovarian cancer were analysed. The tumours comprised a range of histologies: 3 were mucinous, 3 endometrioid, 3 clear cell, 15 serous and 1 of mixed histology. There were 4 Stage 1, 2 Stage 2, 16 Stage 3 and 3 Stage 4 invasive adenocarcinomas. All patients were staged at laparotomy, in accordance with the recommendations of the International Federation of Gynaecology and Obstetrics (FIGO). Germline (from peripheral blood) and tumour DNA was isolated as described previously (Chenevix-Trench et al, 1992).

Polymerase chain reaction (PCR)

PCR primers for the amplification of genomic DNA were either designed using the DNASTAR Lasergene programme or, in the case of TGFβ1 exon 7b (Cardillo and Yap, 1997) and TGFβRII (Vincent et al, 1996), derived from the literature (Table 1). Exons 8, 9, 27, 28, 31, 33, 34, 40, 47 and 48 of IGFRII, exons 5, 6 and 7 of TGFβ1 and exons 3, 5 and 7 of TGFβRII were examined. Standard reaction mixtures contained 0.2 mM dNTPs, 0.2 μM primers, 1 × PCR buffer, and 2.5 units Qiagen *Taq* DNA polymerase. Primer pairs were generally optimized by systematically varying annealing temperatures and number of cycles for standard and 'touchdown' PCR protocols (Roux, 1995), as well as by varying final concentrations of MgCl₂ and the addition of Q solu-

Table 1 PCR primers: sequences and sizes of PCR products

Gene	Exon	Forward primer 5'→3'	Reverse primer 5'→3'	Product size
IGFIIR	8	TTTTTATTTGCTTCTTTAC	ACTTGGCCCCTACTGTCC	278
	9	GCTGTAGTTTGGGCTGGTGTTC	CCGGAGGCTCTGATTGTGGT	285
	27	AAGGGGACAACGTGTGAGGTGAA	TGCAACAAAAGGAAAACGACAAA	252
	28	GGGAGGCCAGGGATACTTTGTC	AGCAGCCTGAGGGTGGGAAGAA	305
	31	AGTTCTGTTCTAGCCTGGGGAGTC	CAGGTGAATCGGATGGTGGTTGA	235
	33	GTCCCATCCTCCACCCTACA	TTCTGGCTCTGCTCATCCTTTTT	249
	34	TGTTTTCTCCGCCTTTCCCTTGTC	TCTCCCGTGCCCATCTGAAAACCTC	313
	40	CTACCGGACATCCAGCATCATA	GAGCCAACCATCGTCAAGCAGTCT	305
	47	TTGGGTGAGTTTTGTGGGGTTTTA	ACGGGTGTTGCTTTCTTTCTG	303
	48	GGGCTCACGTGGTCTCTGCTGTT	CATTGCTCTGTGCGTTTCTCA	294
	TGFβ1	5	TCCCCTATCCCCTGACTCCACAC	CTCCATCCAGGCTACAAGGCTCAC
6		GCCCCTCCCTGCCCTGAT	ACCTTGCTGTACTGCGTGTCC	194
7a		GCCCGCCGCCCGCAGGTC	GAGTGGGGGAACGTCAGGGATGGA	374
7b		ACGAGCCTGAGCCCTGA	CAGGTCGCCCTGTACAA	203
TGFβRI	5	GCCCAACCGAAATGTTAATTC	GGTAGAACTGCTTATAGAAT	221
TGFβRII	3	TGCAATGAATCTCTTCACTC	CCCACACCCTTAAGAGAAGA	240
	5	GGCAGCTGGAATTAATGATGGGC	TGCTCGAAGCAACACATG	255
	7	CCAACTCATGGTGTCCCTTTG	TCTTTGGCAATGCCAGCCTG	250

tion. Positive (β -globin) and negative (no template DNA) controls were included in each PCR run, and amplification of a single band was confirmed by agarose gel electrophoresis and ethidium bromide staining.

Single-stranded conformation polymorphism (SSCP) analysis

The PCR products were diluted 1/10 with sterile water. Equal volumes of diluted sample and 2 \times formamide loading buffer were heated to 95°C for 3 min to denature the samples, and immediately placed on ice to prevent DNA strands from reannealing. A 2 μ l aliquot of each sample was electrophoresed alongside non-denatured and denatured controls. Altogether 4 different SSCP conditions were used, with polyacrylamide gels consisting of 8% or 14% polyacrylamide, with or without glycerol (10%). Setting agents were 15 μ l 25% (w/v) ammonium persulphate and 15 μ l TEMED for every 10 ml of non-denaturing gel. Electrophoresis was performed at room temperature (20–24°C) using 0.5 \times TBE running buffer. Mini gels were electrophoresed at 40 mA for 10 min, then typically for 50 mA for 2 h. The DNA was visualized using silver staining in standard conditions. The gels were washed, transferred to Whatman 3 mm filter paper, dried and stored.

DNA sequencing

Tumour samples that gave abnormal SSCP mobility shifts were re-amplified by PCR from the original DNA sample and purified with the Wizard PCR Preparations DNA Purification System (Promega). Genomic DNA from blood samples were also sequenced, to confirm that any observed sequence variation was tumour specific. Sequencing was performed with the forward primer using an Applied Biosystems Model 377 automated sequencer. Any putative mutations identified by DNA sequencing were confirmed by re-sequencing a sample of the original DNA with forward and reverse primers.

RESULTS

PCR primers for the IGF1R gene were designed to cover the exons that have been reported to date to contain somatic mutations (exons 27, 28, 31, 40, and 48) (IGF1R mutation database: <http://www.geneimprint.com/>), and for those exons for which flanking intronic sequences were available (exon 8, 9, 33, 34 and 47). PCR primers for the TGF β 1 gene were designed to cover the coding region of the mature protein product, namely exons 5, 6 and 7. In the case of exon 7, primers described in the literature were also used (Cardillo and Yap, 1997). In the case of the TGF β R2 gene, exons 3, 5 and 7 were targeted for analysis because they have been found to be mutation hotspots (Vincent et al, 1996).

SSCP analysis was carried out on 25 tumour and normal

samples. TGF β R2 was found to contain point mutations in 3 tumours, but not in the corresponding germline DNA (Figure 1, Table 2). The mutations were all in exon 7, and all 3 were missense mutations in stage 3 tumours. No mutations were found in IGF1R or TGF β 1. A significant association was identified between TGF β R2 mutation status and histology (Fisher's exact test, $P = 0.029$). Of the 3 mutations identified, 2 of were found in 3 clear cell tumours examined, but in only 1 (a serous tumour) out of 22 tumours of other histological types that were examined.

DISCUSSION

It has been proposed that the IGF1R, TGF β 1, and TGF β R2 genes act as a functional unit to negatively regulate cell proliferation. Somatic loss of function mutations in any one of these genes could lead to disruption of the TGF β growth inhibitory pathway and loss of cell cycle control. As mutations in any one gene within the functional unit would be sufficient to disrupt this pathway, there would be little selective pressure to mutate the other genes and so mutations would be expected to be mutually exclusive in individual cancers (Haber and Fearon, 1998).

We have identified somatic mutations in the TGF β R2 gene in 3 out of 25 (12%) tumours but no mutations were found in the IGF1R or TGF β 1 genes. The TGF β R2 amino acid sequence is highly conserved between humans, mice and chickens and all 3 missense mutations occurred at conserved residues (Table 3). The Glu Δ ⁵¹⁵Asp substitution results in a conservative amino acid change and occurs within the functionally important kinase domain, where point mutations have been previously identified (Lynch et al, 1998; Garrigue-Antar et al, 1995). The Arg Δ ⁵²⁸Pro substitution also occurs within this kinase domain, resulting in a non-conservative amino acid change, replacing a positively charged residue with an apolar, uncharged one. X-ray crystallography of other serine/threonine kinases suggests that this highly conserved arginine and preceding conserved glutamate are essential for kinase function (Zhang et al, 1994). The Asn Δ ⁵⁶⁴Tyr

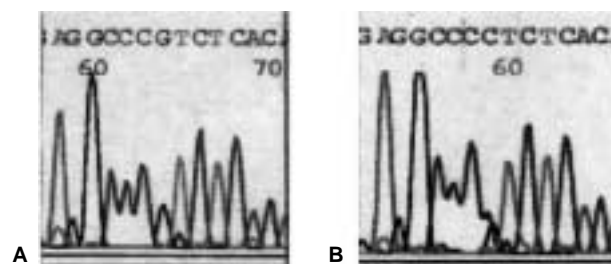


Figure 1 DNA sequence electropherogram. Panels A and B are sequence electropherograms from normal and tumour DNA from sample 14. It shows a region of exon 7 of the TGF β R2. The sequence indicates a CGT to CCT nucleotide change

Table 2 Somatic mutations of TGF β R2 identified in ovarian carcinomas

Gene	Exon	Sample	Nucleotide changes	Amino acid change	Domain	Comment
TGF β R2	7	19	GAG→GAC	Glu Δ ⁵¹⁵ Asp	Kinase	Conservative mutation
	7	14	CGT→CCT	Arg Δ ⁵²⁸ Pro	Kinase	Non-conservative mutation
	7	8	AAC→TAC	Asn Δ ⁵⁶⁴ Tyr	Adjacent kinase	Non-conservative mutation

Three missense mutations were identified in the TGF β R2 gene.

Table 3 Conservation of TGF β RII protein sequences between human, mouse and chicken. The mutant sequence shows the human exon 7 TGF β RII sequence from residue 509–566, with the 3-point mutations identified in human ovarian carcinomas highlighted by shading. The amino acid positions that are not conserved in the mouse or chicken, compared to human protein sequence, are highlighted by shading

TGF β RII type	Protein sequence
Mutant	GIQMVCDTLTECWDHDPPEARLTAQCVAERFSELEHLDRLSGRSCSEEEKIPEDGSLYTT
Human	GIQMVCELTTECWDHDPPEARLTAQCVAERFSELEHLDRLSGRSCSEEEKIPEDGSLNNT
Mouse	GIQIVCETLTTECWDHDPPEARLTAQCVAERFSELEHMDRLSGRSCSQEKIPEDGSLNNT
Chicken	GIQMVCELTTECWDHDPPEARLTAQCVAERFSEFKHHDKLSGRSCSEEEKIPEDGSLVTT

substitution occurs outside the kinase domain, but also results in a non-conservative amino acid replacement, exchanging a polar uncharged residue to an aromatic non-polar one. These types of changes are well known to affect the protein folding of many kinases, often leading to changes in kinase activity or substrate recognition (Taylor et al, 1992). Functional assays would be necessary to confirm the consequences of these mutations, but given their nature and location it is likely that they contribute to tumorigenesis.

TGF β RII mutations were significantly associated with histology, and mutations occurred in 2 of the 3 clear cell carcinomas examined. This type of ovarian tumour represents a distinct histological type where the overall prognosis for patients is poor in comparison with other histologies. The outcome is largely due to the poor response to conventional platinum-based chemotherapy such as cisplatin (Goff et al, 1996; Makar et al, 1995; Tanmella et al, 1998).

This study identified tumour-specific TGF β RII sequence alterations in 3 out of 25 (12%) patient samples. Lynch et al (1998), who performed SSCP analysis of the entire coding region of the TGF β RII gene, found TGF β RII mutations in 6 out of 24 (25%) epithelial ovarian carcinomas. All 4 missense mutations reported by Lynch et al (1998) resulted in non-conservative amino acid substitutions. The relevance of these figures to ovarian carcinoma overall is unclear because both studies examined relatively small numbers of tumours, and SSCP has a sensitivity level of approximately 70% in detecting mutations (Orita et al, 1989). This is of particular concern if specific common, missense mutations are refractory to detection by SSCP. In addition, we chose to screen known gene hotspots, and it is possible that mutations are located within unscreened exons. Therefore the observed mutation frequencies should be regarded as minimum estimates. Lynch et al (1998) also performed immunohistochemistry (IHC) to examine the expression of TGF β RII protein. A total of 15 out of 25 (62%) samples demonstrated complete loss or a significant decrease of TGF β RII gene expression. This data suggests that factors other than mutations in the coding region of this gene may contribute to loss of protein expression, and thus to tumorigenesis. Such mechanisms could include promoter hypermethylation, genomic mutations outside the coding region affecting transcriptional activation or errors in post-translational modification.

We were unable to identify mutations in exon 5 of the TGF β R1 gene in 11 ovarian cancer cell lines by direct sequence analysis, and 20 tumour samples by SSCP. This variant causes a significant change in DNA sequence, which should have been detected by SSCP or DNA sequence analysis. The discrepancies between our study and Wang et al (2000) remain unexplained. As larger studies emerge, correlations between specific histology and mutations in each of the genes may be seen.

Other members of the TGF β functional pathway may also be targets of mutation since other proteins, in addition to IGFIIR, TGF β 1, TGF β R1 and TGF β R2, are required. Candidates would

include Smad2, Smad4 and p21, which are all mutated in various types of cancer (ten Dijke and Heldin, 1999). However, the recent study by Wang et al (2000) would suggest that mutations in Smad2 and Smad4 are rare. While mutations occur relatively frequently in various members of the pathway, there is currently little data to determine whether there is also decreased gene expression of these genes, due to epigenetic mechanisms such as promoter hypermethylation, as has been seen for other tumour suppressor genes. Examination of key components of the TGF β functional pathway in a large series of patient samples would provide vital information regarding the importance of this pathway in the progression of ovarian cancer. Demonstration of a relationship between TGF β RII mutations and clear cell carcinomas of the ovary would suggest that the TGF β pathway is important in the development of this distinct and aggressive subtype of epithelial ovarian carcinoma. The possibility of distinct developmental pathways for epithelial ovarian cancer subtypes has been suggested previously. Somatic mutations in the PTEN (Obata et al, 1998) and β -catenin (Wright et al, 1999) genes are found most frequently in endometrioid ovarian tumours, suggesting that these genes may promote differentiation toward this specific subtype. The results presented here provide further evidence for the role of distinct developmental pathways in the progression of epithelial ovarian cancer subtypes.

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