Expression of a novel mRNA in human head and neck squamous cell carcinoma cells

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Abstract

Aims—The differential display reverse transcription polymerase chain reaction (DDRT-PCR) technique was used to search for differences between the mRNA expression profiles of squamous cell carcinoma (SCC) cell lines established from head and neck tumours and normal keratinocytes from the mucosa of the upper aerodigestive tract.

Methods—Total RNA prepared from both cell types was reverse transcribed into cDNA then amplified in a PCR mixture. To compare the electrophoretic patterns, mRNAs were amplified by nested PCR using specific oligonucleotides. Additionally, using labelled cDNA probes, northern hybridisation was carried out on three cancer cell lines of different origin, a biopsy from a parotid gland pleomorphic adenoma, healthy mucosa, and keratinocytes.

Results—Comparison of the separated bands revealed a fragment with a differential expression pattern in the SCC cells. This cloned sequence of a 336 base pair mRNA fragment exhibited no significant homology with known transcripts. Additionally, after amplification and sequencing of the 3' end of the fragment no homology with a known human gene sequence was found. However, low homology with a genomic sequence of a nematode was found. Northern hybridisation confirmed the selective expression of this fragment in SCC cells versus the cancer cell lines of different origin, the biopsy of the pleomorphic adenoma, keratinocytes, and healthy mucosa.

Conclusions—This is the first differentially expressed human genome transcript of squamous cell carcinoma of the head and neck identified by DDRT-PCR. It may prove useful, in the future, to characterise this tumour type.

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Keywords: squamous cell carcinoma; mRNA expression; differential display reverse transcription polymerase chain reaction

tification of transcripts present in one sample but not in another.⁸⁻¹⁰ However, these methods are difficult to set up, non-reproducible, and they require large amounts of RNA.¹¹ In 1992 Liang and Pardee,¹² as well as Welsh *et al*,¹³ developed a technique called differential display, or RNA based arbitrarily primed PCR, to improve and accelerate the identification of differentially expressed genes. For mRNA containing a poly(A) sequence at the 3' end, oligo(dT) nucleotides were used to prime cDNA synthesis. This method was subsequently refined by introducing non-denaturing gels and establishing the appropriate numbers of optimal PCR primers. This improved technique was named differential display reverse transcription polymerase chain reaction (DDRT-PCR).¹⁴ Using this technique, selectively expressed RNA transcripts have been identified in various organisms¹⁵⁻¹⁷ and in different human tumours.¹⁸⁻¹⁹ Because the identified fragments can be used for detection and isolation of complete mRNA and cDNA, this method has become an increasingly used, alternative means for rapid analysis of the selective expression of genes involved in the pathogenesis of various diseases.

Applying the DDRT-PCR technique, we studied squamous cell carcinoma (SCC) cells of the larynx and the floor of the mouth, as well as corresponding normal keratinocytes, to identify and characterise their differential expression of mRNA. We also used specific probes to identify complementary RNA fragments expressed in SCC cells and tumour biopsies.

Methods

CARCINOMA CELLS AND TUMOUR BIOPSIES

Human head and neck SCC cell lines were studied. Two of the cell lines, UM-SCC-17A and 14B, had been established and characterised by Carey *et al*,²⁰ the cell line, Hlac-78, by Zenner *et al*,²¹ the cell line, HCFMK-1, by Görögh *et al*,²² and normal keratinocytes by Saffran *et al*.²³ The SCC cell lines were grown in minimum essential medium with Earle's salts and L-glutamine, without addition of non-essential amino acids or sodium bicarbonate, but in the presence of 20 mM Hepes buffer and 10% (vol/vol) fetal calf serum (Biochrome, Berlin, Germany) at 37°C in a 5% CO₂ humidified atmosphere. The keratinocyte cultures were grown in serum free medium (Gibco, Eggenstein, Germany) supplemented with 50 mg/ml bovine pituitary extract and 1.5 ng/ml epidermal growth factor in the presence of 100 U/ml penicillin/streptomycin. Bacterial contamination of the cultures was

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The understanding of malignant diseases has grown in recent years, particularly as a consequence of extensive analysis of gene expression. A number of genes affecting nuclear and cytoplasmic functions have been shown to play an important role in the malignant transformation of cells.¹⁻⁷ Various hybridisation procedures are used for the iden-

screened by DNA staining as described by Russell *et al.*²⁴ and by inoculation of the culture medium at 37°C with and without cells.

Two carcinoma biopsies, five normal upper aerodigestive tract mucosa samples, one biopsy of a parotid gland pleomorphic adenoma, RNA from an adenocarcinoma cell line of the lung (HTB-179), the malignant melanoma cell line (HTB-66), and the breast carcinoma cell line (HTB-20) (ATCC, Rockville, USA) were used for northern blotting. The cell lines were grown under the conditions described above. One of the two tumour biopsies subjected to hybridisation was a moderately differentiated SCC of the glottis, the other was a poorly keratinised SCC from the pyriform fossa.

ISOLATION OF TOTAL RNA

For rapid and reproducible preparation of total RNA, the Rneasy Kit (Qiagen, Hilden, Germany) was used in accordance with the manufacturer's instructions. After determination of the RNA content using a photometer (Uvicon-931 UV; Kontron, Hamburg, Germany), samples of total RNA were adjusted to 3.5 µg for subsequent first-strand cDNA synthesis. Quantitative analysis of the mRNA content was carried out using RT-PCR of actin as a control.

FIRST-STRAND CDNA SYNTHESIS

Total RNA (3.5 µg) was heat denatured (65°C, 10 minutes), chilled on ice and subjected to oligo(T) primed reverse transcription using a first-strand cDNA synthesis kit (Pharmacia, Freiburg, Germany). Reverse transcription in a final volume of 33 µl was performed at 37°C for 60 minutes in the presence of 0.2 µM of one of 12 different dodecameric anchored oligo(T) primers (dT₁₀MN; A, G or C) and 40 units of RNase inhibitor (RNasin; Gibco). Following synthesis of the completed first-strand cDNA, the resulting double-stranded RNA:cDNA heteroduplex was heat denatured at 95°C for five minutes to provide cDNA as a template for polymerisation.

mRNA DIFFERENTIAL DISPLAY

The first-strand cDNA product (160 µg) was incubated with 0.2 mM of one of 26 decameric arbitrary primers,¹⁴ 0.2 µM of the corresponding anchored oligo(T) primer, 2.5 µM dNTPs, 1.5 mM MgCl₂, and 2.5 U Taq polymerase in a total volume of 50 µl. Low-stringency PCR was run for 40 cycles at 95°C for 60 seconds, 40°C for 120 seconds, 72°C for 30 seconds, with a final extension step at 72°C for 10 minutes (Thermocycler 4800; Applied Biosystems, Weiterstadt, Germany). After thermocycling, the amplicons were purified using Qiaquick spin (Qiagen), dried in a vacuum centrifuge, resuspended in 7.0 µl TE buffer²⁵ and run on a 6% non-denaturing polyacrylamide (PAA) sequencing gel in Mops buffer²⁵ at 400 V for three hours under constant cooling at 15°C using a flatbed electrophoresis system (EFB-300; Pharmacia). Gels were stained with silver nitrate according to the method of Bassam *et al.*²⁶

ISOLATION, REAMPLIFICATION, AND SEQUENCING OF DIFFERENTIAL DISPLAY

Bands of interest were excised from the gel and the DNA extracted using the Nucleon Easy-clean kit (Scotlab, Wiesloch, Germany). Four microlitres of the DNA elution volume of 50 µl was subjected to PCR under the same conditions as above, except that the concentration of both primers was reduced to 40 pmol. Routinely, 5 µl of the 50 µl reamplified products was separated and stained as above. Purified double-stranded DNA fragments were ligated into the pGEM-T-cloning vector (Promega, Heidelberg, Germany), cloned, and sequenced on a DNA sequencing apparatus (Abi Prism 310; Applied Biosystems).

ANALYTICAL HIGH STRINGENCY NESTED PCR

After sequencing of the differentially expressed fragment, 5 µl of the first-strand cDNA mixture was subjected to PCR (50 µl) using 2.5 U Taq polymerase, 50 µl PCR buffer (Gibco), 0.2 mM each dNTP, 1.5 mM MgCl₂, and 0.2 µmol of forward and reverse primers. The following primers were used: sense 1, 5'-ATG AAG ATA AAG CAC-3'; antisense 1, 5'-TG GGG GAG GGG GTG-3'; sense 2, 5'-TTG TTG GCC ACC-3'; antisense 2, 5'-GCT TTT TTA CAT-3'. The first PCR was run for 30 cycles with sense 1 and antisense 1 primers and the second for 20 cycles with sense 2 and antisense 2 primers at 94°C for 60 seconds, 58°C for 60 seconds, and 72°C for 120 seconds, with a five minute extension at 72°C. After thermocycling, the amplicons were purified using Qiaquick spin (Qiagen) and 2 µl aliquots of the PCR product were analysed by electrophoresis on a 6% PAA sequencing gel stained with silver nitrate as above.

ONE-SIDED PCR

One-sided or anchored PCR^{27, 28} was carried out to amplify the 3' end of cDNA. In this procedure, RNA was extracted and transcribed into cDNA using reverse transcriptase (RT) and an oligo(dT) adaptor primer (Gibco). The cDNA was then amplified by PCR (50 µl) using 2.5 U Taq polymerase, 0.2 mM each dNTP, 5 µl PCR buffer (Gibco), 0.2 µl sequence specific primer (SSP) (5'-CAGGGCTCCTGCCCGATTG-3') that anneals to a region of the selectively expressed fragment obtained by differential display PCR, and 0.2 µM adaptor primer that targets the poly(A) tail region. The reaction was run for 30 cycles at 94°C for 45 seconds, 55°C for 30 seconds, and 72°C for 90 seconds, with a 10 minute extension at 72°C. Purification, electrophoresis, staining and sequence analysis of the PCR product were carried out as described above.

NORTHERN HYBRIDISATION

The 336 base pair cDNA was random prime labelled using the Dig High prime DNA labelling and detection starter kit II (Boehringer Mannheim, Mannheim, Germany). Twenty micrograms of heat denatured, total RNA from all different cell types and biopsies was separated on 0.8% agarose gels according to

Figure 1 Part of the mRNA expression pattern of laryngeal SCC cells (a) and mucosal keratinocytes (b) using the DDRT-PCR technique. The arrow indicates the differentially expressed 336 base pair band using anchored arbitrary primers. m, molecular weight standards.

Table 1 Nucleotide sequence of the 694 base pair fragment obtained from head and neck squamous cell carcinoma cells

Base pairs	Nucleotide sequence									
1-30	<u>TAC</u>	<u>AAC</u>	<u>GAG</u>	<u>GCC</u>	<u>ATG</u>	<u>AAG</u>	<u>ATA</u>	<u>AAG</u>	<u>CAC</u>	TTC
31-60	CTT	TTG	TTG	GCC	ACC	TGC	ATA	TCC	AAG	GAC
61-90	TCC	AGA	GTA	TCC	AAC	CTT	CAA	AGC	GCT	GTG
91-120	GCT	CAT	TCC	CTG	AAC	AAC	GGT	GCC	AGG	TAC
121-150	TTC	ACA	AAG	CAA	CCC	TCT	GCA	CAC	CAC	AGT
151-180	TTG	CTC	CAG	TCT	CCA	ACA	GAA	TTT	TAC	CTC
181-210	TTG	GCC	TTA	GTA	TTC	ATT	<u>CAG</u>	<u>GGC</u>	<u>TCC</u>	<u>TGC</u>
211-240	<u>CCG</u>	<u>ATT</u>	<u>TGA</u>	<u>ATT</u>	<u>ACT</u>	<u>TTG</u>	<u>TTG</u>	<u>GTT</u>	<u>AAA</u>	<u>AAA</u>
241-270	TGG	<u>TGA</u>	TTT	CTC	<u>TAA</u>	TTC	TAT	AAT	TCA	TTT
271-300	CTG	CTT	TTA	TGG	ATG	GGC	ATT	TTG	ATG	TAA
301-330	AAA	AGC	TCA	TCT	CAC	CCC	CTC	CCC	CAC	CTC
331	GTT	GTA								
337-360			AAG	GAA	GAT	GTA	CTG	AAT	TTC	AAG
361-390	GAG	TCC	GGG	CAT	TTG	TCT	CCT	GTA	TCT	ATC
391-420	AAG	TCA	GAC	TCA	AAG	<u>TAA</u>	GCA	CCA	TAC	TAT
421-450	TTG	TTG	ATC	ATT	TCT	CTC	CCA	CTT	ACC	ACA
451-480	TTT	GCC	TCA	AGC	CAG	GTA	CTA	ACT	CCA	CCT
481-510	AGT	CAC	<u>TAA</u>	CTT	TCC	CTT	GGG	AAC	ACT	<u>TAA</u>
511-540	CCT	GGT	TGT	GAG	<u>TAA</u>	AAA	AAG	AAT	GAA	CAT
541-580	TTG	AGA	TGT	<u>TGA</u>	AAC	AAC	TAT	CGG	GNA	GAT
581-610	GTC	AAT	GAA	CAG	CTC	AAG	ACT	GGA	ATT	AAT
611-640	TCC	ACA	GAG	AAA	<u>TGA</u>	<u>TGA</u>	CTG	AAT	AGT	CAA
641-670	TTT	AGA	<u>AAT</u>	<u>AAA</u>	AAC	TTG	GAC	TTA	AAT	TCC
671-700	AAA	ATG	AAA	AAA	AAA	AAA	AAA	AAA		

The arbitrary 10 base pair primer that was used is underlined. Primers for analytical high stringency nested PCR are highlighted by boldface. 1 sense base pairs 13-27, 1 antisense base pairs 312-325, 2 sense base pairs 34-45, 2 antisense base pairs 294-306. The sequence of the 486 base pair product that was generated using the SSP and oligo(dT) primer is indicated by italics. SSP, as well as start and stop codons, are double underlined.

the method of Sambrook *et al.*²⁵ After ethidium bromide staining of 28S and 18S rRNAs, the gels were subjected to capillary transfer on to a positively charged nylon membrane (Boehringer Mannheim) overnight at room temperature. The transferred RNA was subsequently immobilised by UV irradiation, membranes were prehybridised at 50°C for one hour, and hybridised at 50°C for 14 hours in Easy Hyb solution (Boehringer Mannheim). Membranes were then washed twice at room temperature for 15 minutes per wash, in 0.3 mM NaCl, 30 mM sodium citrate (pH 7.0) containing 0.1% (wt/vol) sodium dodecyl sulphate (SDS), and washed twice again for 15 minutes per wash in 70 µM NaCl, 7.0 mM sodium citrate (pH 7.0) containing 0.1% SDS at 68°C. After stringency washes, the membranes were prepared for chemiluminescent detection with disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5-chloro)tricyclo[3.3.1.1.^{3,7}]decan}-4-yl)phenyl phosphate (Boehringer Mannheim) according to the manufacturer's instructions. Finally, blots were exposed on to x ray film for two to 20 minutes using an intensifier screen.

Results

DIFFERENTIAL EXPRESSION PATTERN

To show the mRNA expression pattern of the cells to be examined, the reverse transcribed cDNA populations were comparatively analysed by DDRT-PCR. Non-denaturing PAA gel electrophoresis could detect selective expression of a 336 base pair mRNA fragment in the head and neck SCC cell line, unlike normal keratinocytes derived from pharyngeal and laryngeal mucosa (fig 1). The reverse transcription step and the PCR reaction for the lane that exhibited a differentially expressed band were repeated and a reproducible difference was corroborated (data not shown). This differentially expressed cDNA fragment was recovered from the gel, reamplified, and cloned into a plasmid vector.

NUCLEOTIDE SEQUENCE OF THE CDNA FRAGMENT

After sequencing of the differentially expressed fragment using both forward and reverse M13 primers, the first 336 bases could be obtained reproducibly. As shown in table 1, the nucleotide sequence begins with a 10mer forward arbitrary primer and ends with the last 10 bases being palindromic to the complementary sequence of the same primer. Because this sequence represented only a part of the complete mRNA, the polyadenylation signal and the poly(A) tail could not be seen. The sequence was read from the 5' to the 3' end in all three possible reading frames and routinely compared with the GenBank database to identify the differentially expressed fragment. In all instances, there was no exact match with any database sequences (including virus, prokaryote, and eukaryote sequences), suggesting the identification of an unknown transcript. However, a sequence region of 54 base pairs showed 63% homology with the human T cell receptor β locus. This was the highest homology reached for this 336 base pair fragment.

Figure 2 Electrophoretic pattern of DNA after amplification with sequence-specific primers by RT-PCR in 5% PAA gel after silver staining. (A) DNA fragment expressed in SCC cell lines corresponding to 314 base pairs produced from the 1 sense and 1 antisense primer combination (table 1). (B) DNA fragment generated in a second PCR corresponding to 273 base pairs from the 2 sense and 2 antisense nested primer combination (table 1). The cells used were: HCFMK-1 (lane 1), UM-SCC-14B (lane 2), UM-SCC-17B (lane 3), and HlaC-78 (lane 4). M, molecular weight standards. (C) Detection of the differentially expressed fragment by non-radioactive northern blot analysis using 20 µg of total RNA per lane after agarose gel electrophoresis and transfer on to nitrocellulose. RNA was isolated from a moderately differentiated SCC of the glottis, from a poorly keratinised SCC of the pyriform fossa, from the UM-SCC-17A, 14B, HlaC-78, and HCFMK-1 cell lines, as well as from laryngeal mucosal biopsies and from cultivated mucosal keratinocytes (lanes 1–8, respectively). Hybridisation was performed with random primed digoxigenin labelled probes specific for the 336 base pair fragment.

NESTED PCR AND NORTHERN HYBRIDISATION

To test the efficiency and specificity of the differentially expressed fragment, high stringency secondary RT-PCR using sequence specific sense and antisense primers was performed. Furthermore, the specificity of the amplification was enhanced with nested primers that annealed within the first primer pair. The differentially expressed fragment generated in the original DDRT-PCR could be amplified as a distinct fragment using both the first primer pair that produced a 314 base pair fragment and the nested PCR primers that produced a 273 base pair fragment, demonstrating the high specificity of the primers to the target sequence (fig 2A and B).

One-sided PCR was carried out to reconstruct the 3' end of the mRNA. Using the 20mer forward SSP and the oligo(dT) primer, a 486 base pair PCR product was generated, encompassing the non-coding region with stop codons (TGA, TAA), polyadenylation signal (AATAAA) and the 3' end poly(A) tail (table 1). The total length of the fragment obtained was 694 base pairs (table 1). Following the forward primer the sequence contained the start codon (ATG) that codes for methionine. Further analysis of the sequence revealed no stop codon within the next 231 base pairs.

This 234 base pair long sequence may represent the coding region. The 3' end amplified part of the mRNA molecule was then compared again with the GenBank database and a homology of 52% with the gene sequence of the nematode worm *Caenorhabditis elegans* was found, involving 198 base pairs of the 486 base pair PCR product.

Total RNA from SCC biopsies and cell lines was subjected to northern blot analysis using the differentially expressed 336 base pair digoxigenin labelled probe (fig 2C). Under the same hybridisation conditions, the probe hybridised to an ~ 0.7 kb mRNA from all tumour biopsies and cell lines tested. The HCFMK-1, UM-SCC-14B, and UM-SCC-17A cell lines derived from poorly to well differentiated SCC of the floor of the mouth, as well as the HlaC-78 cell line derived from a moderately to well differentiated SCC of the larynx, all exhibited a high expression rate. The hybridisation signal in the moderately differentiated SCC biopsies from the glottis and in poorly keratinised SCC biopsies of the pyriform fossa was weaker. No expression was detected in either mucosal biopsies, in mucosal keratinocytes cultivated in vitro, in the three cancer cell lines, or in the pleomorphic adenoma of the parotid gland.

Discussion

Major technological advances in recombinant DNA, cloning, sequencing, and gene amplification methods have facilitated the use of RNA or cDNA probes in all areas of biomedical research. The differential display PCR technique for gene amplification is a powerful tool and has been used in many aspects of molecular biology, in both research and diagnostic laboratories. The amplification of differentially expressed genes has been used successfully for vertebrates,²⁹ and for eukaryotic organisms in general.^{11 30-32}

We applied the DDRT-PCR technique to analyse the gene expression profile of cultivated SCC cells and mucosal keratinocytes. Using a protocol for non-radioactive detection that we had recently modified,²⁷ reproducible cDNA fingerprints were achieved. Compared with differential cDNA library screening or subtractive hybridisation, the differential display technique has several distinct advantages. The procedure is fast, simple, and reproducible.^{29 33} It also has the potential to detect low abundance messages that cannot be detected with conventional hybridisation based approaches.^{11 14 33}

After reverse transcription of mRNA into cDNA with 3' two-based anchor primers and subsequent amplification with arbitrary primers, we detected a large number of transcripts and isolated a cDNA clone-containing sequence that reacted with all SCC samples, as confirmed by northern hybridisation.

Comparative analysis of the nucleotide sequence of the differentially expressed 336 base pair fragment did not reveal any significant homology with sequences in the DNA database. Furthermore, the sequence of the human T cell receptor β locus showed the highest correspondence, with 63.0% overlap-

ping within a small segment of only 54 base pairs. For further characterisation of this differentially expressed fragment, we attempted to identify the protein coding region. In practice, mRNA transcripts have frequently been detected by the differential display procedure, of which nucleotide sequences represent a 10mer arbitrary primer at one end that is matched by a complementary sequence at the other end. This is due to the nature of the DNA sequence—that is, the same 10mer arbitrary primer anneals to two complementary sequences within the same transcript. Thus, such sequences present neither the polyadenylation signal nor the poly(A) tail characteristic of mRNA molecules. This is, indeed, the case for the 336 base pair clone which starts with the 5'-TACAACGAGG-3' primer (table 1). The sequence ends with 10 bases (5'-CCTCGTTGTA-3'), of which the complementary sequence is palindromic (3'-GGAGCAACAT-5') to the 5' to 3' primer. The lack of added A at the 3' end of cDNA fragments detected by differential display is a well documented fact.¹⁴ Because this fragment was lacking the poly(A) tail, we examined the amino acid sequences of all three possible reading frames but did not find homologous sequences in the protein database. Additional 3' amplification was accomplished by one-sided PCR. The result displayed the stop codons as well as the poly(A) signal of the mRNA. GenBank database comparison was repeated, but again revealed no identity with human gene sequences. This finding suggests that the fragment differentially expressed in laryngeal and floor of mouth carcinoma cells represents a novel gene. Additional analyses, including protein expression studies in bacterial expression systems, are in progress to obtain further information regarding the function of this selectively expressed transcript.

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