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Original Paper

Expression Analysis of Genes at 3q26-q27 Involved in Frequent Amplification in Squamous Cell Lung Carcinoma

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Gene amplifications are known to occur frequently in lung cancer. Recently, we identified gene amplifications at 3q26 in squamous cell lung carcinoma (SCC) using reverse chromosome painting. Here, our aim was to analyse the expression of genes which map within the amplified chromosomal region. The genes which were selected for their known function and their potential involvement in tumour development included the genes for ribosomal protein L22 (*RPL22*), butyrylcholinesterase (*BCHE*), glucose transporter 2 (*SLC2A2*), transferrin receptor (*TFRC*), thrombopoietin (*THPO*) and the phosphatidylinositol-3 kinase catalytic alpha polypeptide (*PIK3CA*). While five genes were expressed in the majority of the 17 samples of SCC, the gene for the glucose transporter 2 (*SLC2A2*) was expressed in only three cases, excluding *SLC2A2* as the target gene of the amplification unit. For a subset of tumours, we determined the amplification status of the six genes. The *TFRC*, *PIK3CA*, *BCHE*, *THPO* and *SLC2A2* genes were amplified in several cases, whereas the *RPL22* gene was amplified in only one case. The combined amplification and expression data of this and our previous studies indicate that the amplified region at 3q26 contains several genes that are transcribed in SCC, providing the possibility that several amplified and functionally important genes at 3q26 may be involved in the pathogenesis of SCC. © **1999 Elsevier Science Ltd. All rights reserved.**

Key words: squamous cell lung carcinoma (SCC), gene amplification, gene expression, chromosomal band 3q26-q27
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INTRODUCTION

VARIOUS TYPES of human cancer are characterised by gene amplification including *neu* in breast cancer, *EGFR* and *Gas41* in glioma and *N-myc* in neuroblastoma [1, 2]. Recently developed approaches including comparative genomic hybridisation (CGH) and microdissection-mediated cDNA capture greatly facilitated the identification and cloning of amplified DNA domains which appear to be even more frequent in human cancers than previously predicted [3, 4]. The detection of gene amplification in tumour cells has been shown to be of prognostic significance [5, 6]. Most notably, clinical trials are underway demonstrating that the decision for chemotherapeutic intervention in neuroblastoma can be based solely on *N-myc* amplification [7].

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Recently, cytogenetic studies showed double minutes as cytogenetic manifestations of DNA amplifications in non-small cell lung cancer which accounts for more than 75% of all lung cancers [8]. CGH revealed 22 different amplified domains in small cell lung carcinoma [9]. Southern-blot analysis indicated several amplified oncogenes in lung carcinoma including *c-myc* and *K-ras2* [10, 11]. While the aforementioned gene amplifications were relatively rare in lung cancers, we recently reported amplifications at chromosome band 3q26 in approximately 40% of squamous cell lung carcinomas (SCC) [12]. The first evidence for frequent amplification at 3q26 in SCC stems from reverse chromosome painting [13]. Subsequent studies revealed frequent amplification of the butyrylcholinesterase (*BCHE*) and glucose transporter 2 (*SLC2A2*) genes, both of which are localised within the amplified domain at 3q26 [12].

Here, we further characterised the amplified domain at 3q26 in SCC. Specifically, we analysed the expression of

genes which map within or near the region 3q26. In addition, we analysed the selected genes for amplification in a subset of SCC.

MATERIALS AND METHODS

DNA and RNA isolation

Genomic DNA was isolated from peripheral blood lymphocytes, normal lung tissue and tumour tissue according to standard protocols [14]. In brief, after proteinase K digestion, proteins were extracted using chloroform and high molecular weight DNA was precipitated with isopropanol. Thus, the yield of DNA from bronchoscopies was between 500 ng and 3 μg, whereas the yield of DNA from resected tumours was greater than 5 μg. Total RNA from tumour tissue was isolated according to the Stratagene RNA-Isolation Kit. After homogenisation of the frozen tumour tissue, proteins were removed using chloroform–phenol extraction. Total RNA was precipitated twice with isopropanol and resuspended in diethylpyrocarbonal (DEPC)-treated H_2O. The RNA concentration and integrity was determined using a formaldehyde gel.

Southern blot analysis

For Southern blotting 5 μg genomic DNA was used, as determined by spectrophotometric measurements. DNA was restricted overnight with 50 U *Eco*RI (Boehringer Mannheim, Mannheim, Germany) and separated on a 0.8% agarose gel. After denaturation in 0.4 M NaOH, DNA was transferred to a Nylon membrane. We used a 1300 bp fragment of the transferrin receptor (*TFRC*) gene and a 450 bp fragment of the *SLC2A2* gene as probes. The probes were labelled with ^{32}P using a random primer labelling kit (Boehringer Mannheim). Hybridisation was carried out overnight at 65°C. After washing the membrane three times in phosphate buffer at concentrations of 450, 250 and 150 mM and containing 1% sodium dodecylsulphate (SDS), signals were identified by autoradiography using an intensifying screen [14].

Comparative polymerase chain reaction (PCR)

Comparative PCR was carried out in either a peltier thermal cycler PTC-100 or a PTC-125 personal cycler from M.J. Research Inc. (Watertown, Massachusetts, U.S.A.) as described elsewhere [15]. In brief, we first prepared a DNA master dilution of 5 ng/μl from blood and tumour DNA, as determined by spectrophotometrical measurement. Using the DNA master dilution we then prepared three dilutions of 2, 1 and 0.5 ng/μl of blood and tumour DNA. For calibration purposes 5 μl of both blood DNA and tumour DNA dilutions were used for PCR amplification. First, PCR was performed using primers specific for the mucin 2 gene (*MUC2*) that served as a single copy control [16]. Using *MUC2* primers, the concentration of blood and tumour were adjusted to yield PCR products of equal intensity in both the blood DNA dilutions and the tumour DNA dilutions. The PCR products separated on a 2% agarose gel were stained by Sybr Green 1 dilution (FMC Bioproducts, Rockland, Maine, U.S.A.) according to the manufacturer's manual. After calibration, amplification of genes was monitored using different gene-specific primers. Each PCR was carried out in a total volume of 50 μl with 0.5 μM of each primer, 200 μM deoxynucleotides and 2.5 U *Taq* polymerase (Pharmacia GmbH, Freiburg, Germany) in PCR buffer containing 500 mM KCl and 100 mM Tris-Cl (pH 8). The $MgCl_2$ concentration for the ribosomal protein L22 (*RPL22*) gene was 2 mM; for the thrombopoietin (*THPO*) and the *MUC2* genes was 1.5 mM; for the *BCHE*, the *SLC2A2* and the phosphatidylinositol-3 kinase catalytic alpha-polypeptide (*PIK3CA*) genes was 2.5 mM and for the *TFRC* gene was 0.5 mM. PCR amplification was carried out with 45 sec initial denaturation at 94°C, 45 sec annealing at 58°C for the *RPL22*, *PIK3CA*, *THPO* and *MUC2* genes and 56°C for the *BCHE*, *SLC2A2* and *TFRC* genes. Primer extension was 45 sec at 72°C. The number of cycles was 26 for all genes except for the *SLC2A2* and *BCHE* genes where the number of cycles was 27. Primer sequences were as follows: *RPL22* forward: 5′TCACTCTTGATTGCACCCAC; *RPL22* reverse: 5′GGTGATCTTGCTCTTGCTCC; *BCHE* forward: 5′TTCCACATCTCTGATCCATG; *BCHE* reverse: 5′ACAGCCACCTCTTGGTAGAC; *SLC2A2* forward: 5′AGCCTGTGGAGCCTGTAAG; *SLC2A2* reverse: 5′CACCAATGTCATATCCAAACTG; *TFRC* forward: 5′CTGAGTGTGAGAGACTGGCA; *TFRC* reverse: 5′CTTGATGGTGCTGGTGAAG; *PIK3CA* forward: 5′ACTCTGGATTCCACACTGCAC; *PIK3CA* reverse: 5′GCTATCAAACCCTGTTTGCG; *THPO* forward: 5′TGATGCTTGTAGGAGGTCC; *THPO* reverse: 5′GCTGAGGCAGTGAAGTTTGTC; *MUC2* forward: 5′CATTCTCAACGACAACCCCT; *MUC2* reverse: 5′GCAAGAGATGTTAGCTGCCC; *GAPDH* forward: 5′ATGTTCGTCATGGGTGTGAA; *GAPDH* reverse: 5′GTCTTCTGGGTGGCAGTGAT.

Reverse transcription–PCR (RT–PCR)

Two micrograms of RNA used for RT–PCR were treated with 20 U DNAse I (RNAse-free/Boehringer Mannheim). After DNAse I digestion, 40 ng RNA were used for Alu-PCR to exclude contamination of the RNA by genomic DNA. To exclude the possibility of degradation, the integrity of the RNA was monitored by gel electrophoresis prior to RT. For first strand cDNA synthesis, 1.5 μg RNA was used according to the Stratagene RT–PCR kit instructions, whereas the remaining 500 ng of the DNA-free RNA was used for control purposes in the following RT–PCR experiments. RT was performed using random hexanucleotide primers. After RT, 1/10 of the total reaction volume was used for PCR with the primers for the *RPL22*, *TFRC*, *THPO*, *SLC2A2*, *BCHE* and *PIK3CA* genes. Fifty nanograms of the corresponding DNA-free RNA served as a control. PCR for each primer pair was performed for 29–31 cycles on a peltier thermal cycler PTC-100. The PCR products were separated on a 2% agarose gel and stained with Sybr Green 1 (FMC Bioproducts).

Due to small amounts of RNA, gene expression was analysed by RT–PCR. cDNA of different normal human tissues including lung, liver, muscle and gall bladder served as controls. Prior to expression analysis, the quality of the RNA was tested by gel electrophoresis and the efficacy of the RT was verified using primers for the glycerinaldehyde-3-phosphate-dehydrogenase (GADPH) gene.

Genes analysed

Genomic DNA and RNA were isolated from samples of 17 SCC that were either derived by bronchoscopy or surgically resected and processed for amplification and expression analysis of genes previously located at chromosomal band 3q26-3q27. The genes tested for amplification and expression included *RPL22* at chromosomal band 3q26, *BCHE* at band 3q26.1-q26.2, *SLC2A2* at band 3q26.1-q26.3, *TFRC* at band 3q26.2, *PIK3CA* at band 3q26.3 and *THPO* at band

Table 1. *Tumour stage, according to TNM classification, and tumour grade of the specimens used for amplification and expression analysis of genes located at chromosomal band* 3*q*26-*q*27 *in squamous cell lung carcinoma (SCC)*

Tumour sample	TNM staging	Tumour grading
SCC1	T2 N0	III
SCC2	T1 N0	II
SCC3	T2 N1	II
SCC4	T1 N0	II
SCC5	T2 N0	II
SCC6	T2 N0	II
SCC7	T2 N0	II
SCC8	T1 N0	III
SCC9	T3 N1	III
SCC10	T2 N1	II
SCC11	T1 N1	II
SCC12	T3 N2	II
SCC13	T2 N0	III
SCC14	T2 N1	II–III
SCC15	T2 N1	III
SCC16	T2 N0	II
SCC17	T2 N1	II–III

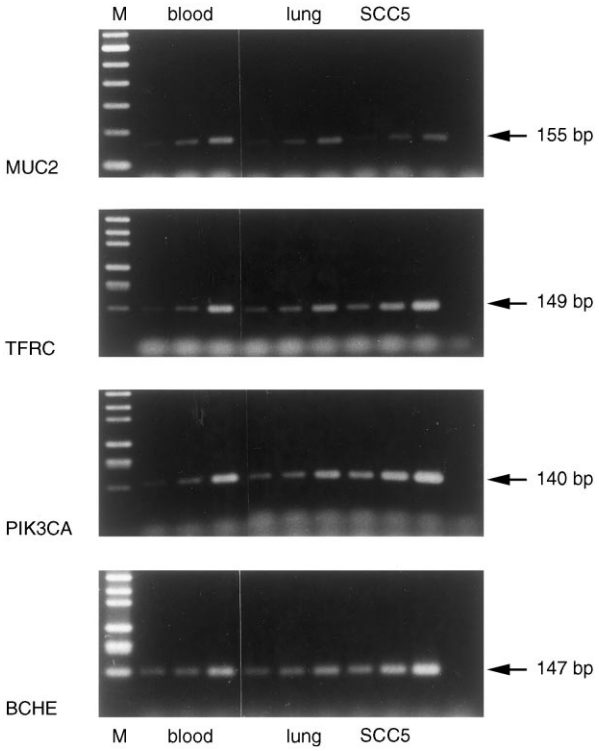

Figure 1. Amplification analysis by comparative polymerase chain reaction (PCR). Three DNA dilutions of blood, normal lung tissue or tumour SCC5 containing adapted amounts of DNA were amplified by PCR using different sequence-specific primer pairs. The increase in signal intensity of the PCR products compared with blood and lung tissue in all three DNA dilutions of tumour SCC5 indicates an increased copy number of the genes *TFRC*, *PIK3CA* and *BCHE*, whereas no increase in gene copy number in lung tissue was observed. Lane 1, marker VI (Boehringer Mannheim) or 100 bp molecular weight ladder, respectively (Gibco); lanes 2–4, three dilutions of blood DNA; lanes 5–7, three dilutions of DNA from lung tissue; lanes 8–10, three dilutions of DNA from tumour SCC5.

3q26.3-q27. These genes were selected for their potential involvement in tumorigenesis, specifically for their involvement in signal transduction and cell growth control.

A subset of eight tumours was tested for amplification of the selected genes. For small tumour samples that yielded only small amounts of DNA, we used comparative PCR which was previously shown to allow a highly reliable determination of gene copy number changes [12, 15]. The *MUC2* gene which maps at 11p15.5 was used for calibration purposes.

RESULTS

Prior to DNA and RNA isolation, the SCC tissue was analysed by the Department of Pathology at the University of Saarland to ensure that only tumour tissue was used in the following experiments. Table 1 summarises the data concerning the tumour stage, according to TNM classification, and the tumour grade of the specimens used for analysis.

Amplification of the *TFRC*, *PIK3CA*, *BCHE*, *THPO* and *SLC2A2* genes was observed in several cases, whereas

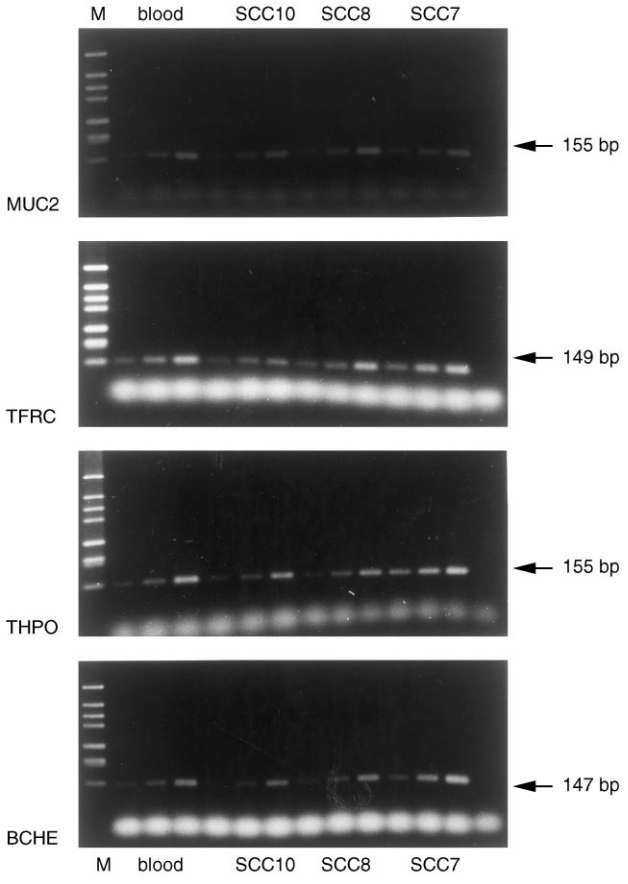

Figure 2. Amplification analysis by comparative polymerase chain reaction (PCR). Three different dilutions of DNA from blood or tumours SCC7, SCC8 and SCC10 were amplified by PCR using gene-specific primers and the PCR products were separated by gel electrophoresis. The stronger signal intensity of the PCR products compared with blood in all three DNA dilutions of tumour SCC7 indicates an increased copy number of the genes *TFRC*, *THPO* and *BCHE*, whereas no increase in gene copy number was detected in tumours SCC10 and SCC8. Lane 1, marker VI (Boehringer Mannheim); lanes 2–4, three dilutions of blood DNA; lanes 5–7, three dilutions of DNA from tumour SCC10; lanes 8–10, three dilutions of DNA from tumour SCC8; lanes 11–13, three dilutions of DNA from tumour SCC7.

amplification of the *RPL22* gene was observed in only one case. Examples of the amplification analysis by comparative PCR are shown in Figures 1 and 2. In cases with sufficient tumour DNA, Southern blot hybridisation was performed using probes of the *SLC2A2* and *TFRC* genes to evaluate the amplification status. Examples of the amplification analysis by Southern blot are demonstrated in Figure 3. The results of the Southern blot hybridisation were consistent with the data obtained by comparative PCR showing an amplification of the *SLC2A2* and *TFRC* genes.

An example of the RT–PCR using GAPDH primers as a control is given in Figure 4. The majority of the analysed genes were expressed in all control tissues except for the *TFRC* and *SLC2A2* genes which were not expressed in muscle. The *RPL22*, *BCHE*, *TFRC*, *THPO* and *PIK3CA* genes were expressed in the majority of SCC. In detail, the *RPL22* and *TFRC* genes were expressed in 17 tumours, *PIK3CA* in 16, *BCHE* and *THPO* in 15, and *SLC2A2* in only 3 tumours.

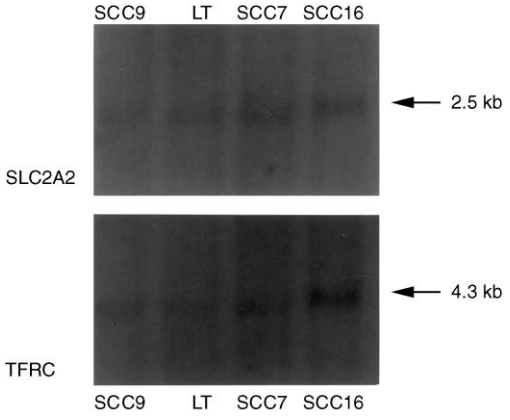

Figure 3. Gene amplification analysis by Southern blot hybridisation. DNA from tumours SCC7, SCC9, SCC16 and normal lung tissue (LT) were *Eco*RI restricted and hybridised with ^{32}P labelled probes of the *SLC2A2* and *TFRC* genes. The small increase in signal intensity is indicative of an increase in gene copy number of the corresponding gene.

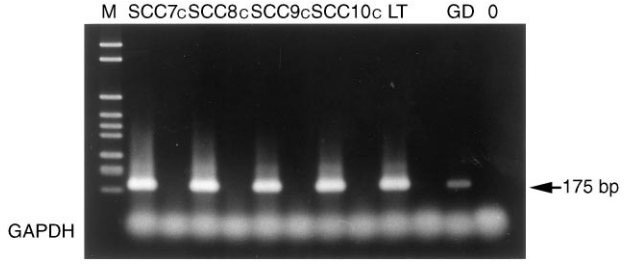

Figure 4. Reverse transcription–polymerase chain reaction (RT-PCR) to evaluate the efficacy of RT and cDNA integrity using glycerinaldehyde-3-phosphate-dehydrogenase (GADPH) control primers. cDNA of different squamous cell lung carcinomas (SCC) and the corresponding DNAse-treated RNA that served as a negative control were used as templates. Lane 1, molecular weight marker VI (M; Boehringer Mannheim); lanes 2–3, cDNA and corresponding DNAse-treated RNA (c) of SCC sample SCC7; lanes 4–5, cDNA of sample SCC8 and corresponding RNA; lanes 6–7, cDNA of sample SCC9 and corresponding RNA; lanes 8–9, cDNA of SCC sample SCC10 and corresponding RNA; lanes 10–11, normal lung tissue (LT) and corresponding RNA; lane 12, genomic DNA (GD) that served as a positive control; lane 13, PCR master mix without any template (0) that served as a negative control.

Examples of the expression analysis by RT–PCR are shown in Figure 5.

DISCUSSION

Recently, we reported the amplification of the *BCHE* and *SLC2A2* genes at chromosome band 3q26 in SCC [12]. In the present study, we extended the analysis of the amplification unit at chromosomal band 3q26 to additional genes. Location at 3q26-q27 was confirmed for all the genes tested according to the Genome Database (GDB) except for the *TFRC* gene. This gene was formerly localised at chromosomal band 3q26.2. Other studies indicate that the *TFRC* gene is located at 3q29 [17].

Specifically, we analysed the expression of the additional genes to gain further insight into the biological significance of the amplification unit at 3q26. Unfortunately, only small amounts of RNA were available, limiting the expression analysis to RT–PCR. Except for the *SLC2A2* gene, the genes were expressed in the majority of tumours and control tissues. Previously, we identified *SLC2A2* as frequently amplified in SCC [12]. The lack of expression shown in the present study excludes the *SLC2A2* gene as the target gene of the

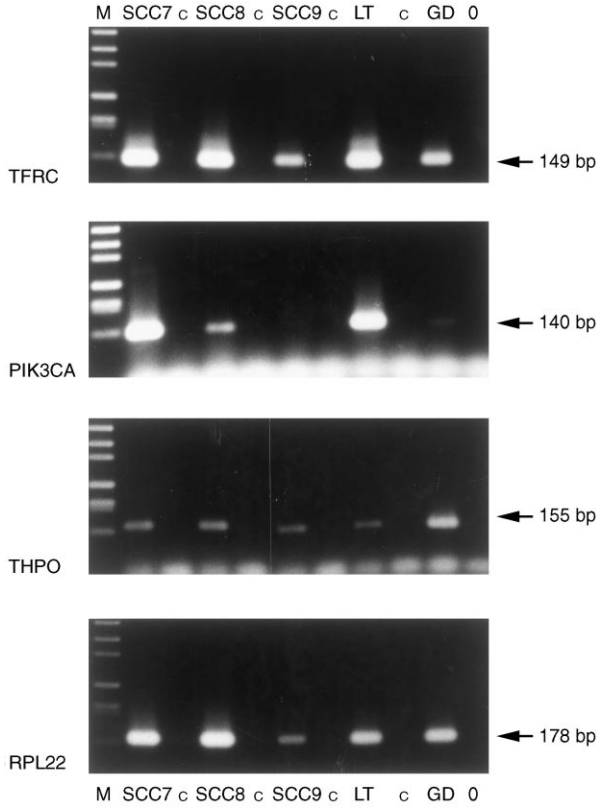

Figure 5. Expression analysis of squamous cell lung carcinoma (SCC) samples using reverse transcription–polymerase chain reaction (RT–PCR). DNAse treated RNA from different SCC was reverse transcribed by random priming and used for PCR amplification with primers specific for the *TFRC*, *PIK3CA*, *THPO* and *RPL22* genes. Lane 1, molecular weight marker VI (M; Boehringer Mannheim); lanes 2–3, cDNA and corresponding DNAse-treated RNA (c) of SCC sample SCC7; lanes 4–5, cDNA of sample SCC8 and corresponding RNA; lanes 6–7, cDNA of sample SCC9 and corresponding RNA; lanes 8–9, cDNA of normal lung tissue (LT) and corresponding RNA; lane 10, genomic DNA (GD) that served as a positive control; lane 11, PCR master mix without any template (0) that served as a negative control.

amplification unit. As for the remaining genes, no conclusion about a possible target gene function can be deduced from the expression data.

By employing comparative PCR or Southern blot hybridisation, we analysed the amplification level in selected tumour samples. Using comparative PCR, even slight changes in gene amplification status can be monitored in comparison with Southern blot hybridisation. Four of eight SCC showed amplification for at least one of the analysed genes. The overall frequency of amplification events at 3q26 in SCC is consistent with our previous studies showing gene amplification at 3q26 in more than 30% of SCC. Unfortunately RT–PCR does not allow a reliable quantification of the gene expression levels to correlate amplification with expression.

Based on their functions, several of the analysed genes mapped at 3q26-q27 may play a critical role in the pathogenesis of SCC. The transferrin receptor, expressed on the cytoplasmatic membrane in a wide variety of human cells, is thought to play a crucial role in the control of cell growth [18, 19]. Previous studies have demonstrated expression of the transferrin receptor in the majority of bronchogenic carcinomas, including SCC [20]. These tumours have also been shown to express transferrin that can act as an autocrine promoter of cellular proliferation as other growth factors in malignant cells [21, 22]. Phosphatidylinositol-3 kinase was found as a major phosphothyrosyl protein subsequent to growth factor stimulation and oncogenic transformation [23] and is involved in several cell signalling pathways, including those of epidermal growth factor (EGF), platelet derived growth factor (PDGF) and insulin-like growth factor (IGF) [24, 25]. RPL22 is a cellular 15 kDa ribosomal protein that has been previously shown to bind the Epstein–Barr virus (EBV) small RNA EBER1 in EBV-infected B lymphocytes [26]. The *RPL22* gene has also been identified as the target of a chromosomal translocation in leukaemia patients [27]. Amplification and expression of the butyrylcholinesterase gene (*BCHE*) has been reported for different tumours, including leukaemia, brain and ovarian cancer [28]. However, no precise function of *BCHE* has yet been established. The glucose transporter 2 gene (*SLC2A2*) is known to play a crucial role in glucose metabolism and might be involved in the pathogenesis of head and neck tumours [29]. Furthermore, our expression data strongly suggest that *SLC2A2* is not relevant in the pathogenesis of SCC. The thrombopoietin gene (*THPO*) plays a crucial role in the growth and differentiation of megakaryocyte-lineage cells. Thrombopoietin is a physiologically regulated cytokine capable of supporting different aspects of megakaryopoiesis [30].

In summary, our study demonstrates the expression of six genes localised within the amplified chromosomal region 3q26. Their specific function in either signal transduction or cell growth control suggests that some of these genes might be the target genes of the amplification unit playing a crucial role in the pathogenesis of SCCs. Particularly the *TFRC* and *PIK3CA* genes, which are expressed in the majority of the SCC samples and amplified in several cases, are good candidates for further analysis concerning their role in tumorigenesis.

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