[CANCER RESEARCH 57, 2290–2294, June 1, 1997]

Amplification of the Genes *BCHE* and *SLC2A2* in 40% of Squamous Cell Carcinoma of the Lung[1]

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ABSTRACT

Gene amplification is a common genetic change in human cancer cells. Previously, we provided the first evidence for gene amplification at chromosome band 3q26 in squamous cell lung carcinoma. In this study, the following analyses were performed: (*a*) we evaluated biopsies and paraffin-embedded tissues of 16 additional squamous cell lung carcinomas for gene amplification using reverse chromosome painting. Of the 16 tumors, 3 tumors showed an amplification of the entire long arm of chromosome 3, and 3 tumors showed various amplifications on 3q, all of which involved chromosome band 3q26; (*b*) we tested eight genes encompassing region 3q25–qter in two different tumors to identify amplified genes on chromosome 3q. The genes *SI*, *BCHE*, and *SLC2A2* were amplified in both tumors; and (*c*) we analyzed 15 additional paraffin-embedded tissues to determine the amplification frequency of these genes. Of the 15 squamous cell lung carcinomas, 6 showed amplification for at least 1 of the genes, with *BCHE* and *SLC2A2* as the genes most frequently amplified. Together, our reverse chromosome painting data and our PCR analysis indicate gene amplification at 3q26 in 40% of all squamous cell lung carcinomas with *BCHE* and *SLC2A2* as possible target genes of the amplification unit in squamous cell lung carcinoma.

INTRODUCTION

There is an increasing number of tumor-related deaths that can be attributed to carcinomas of the lung (1). Several environmental factors, most notably tobacco smoke, play an interactive role in the genetic changes that occur during the pathogenesis of lung carcinoma (2). Although the cytogenetic data on lung cancer are somewhat limited, complex cytogenetic changes are reported in several studies (3, 4). The identification of nonrandom chromosomal alteration has indicated an involvement of several oncogenes and tumor suppressor genes in lung carcinoma. Mutations have been reported for a variety of genes including genes of the *RAS* family, the *Rb* gene, and the *p53* gene (5–7). The genes *CMYC* and *KRAS2*, the topoisomerase II gene, and the gene for parathyroid hormone-related protein have been found to be amplified in lung carcinoma (8–11).

Gene amplification has been shown to occur in numerous human tumors and was proposed to contribute to tumorigenesis (12, 13). The identification of double minutes in non-small cell lung cancers, which accounts for 75–80% of all lung tumors, provided further evidence for a prevalent role of gene amplification in lung cancers (14). Amplification of specific genes, however, has only been detected at a relatively low frequency in lung cancer.

Most recently, we reported DNA amplifications on chromosome band 3q26 in 30% of squamous cell lung carcinoma (15), providing the first evidence for a particular region to be frequently amplified in squamous cell lung carcinoma. This analysis, however, was performed on a limited number (n = 9) of tumors. In the present study, we set out to provide further evidence for the frequent involvement of chromosome region 3q26 in amplification events in squamous cell lung carcinoma. In detail, we performed: (*a*) RCP[3] of additional squamous cell lung carcinoma; (*b*) comparative PCR and Southern blot analysis of genes localized at 3q25–qter; and (*c*) analysis of the amplification frequency for selected genes.

MATERIALS AND METHODS

Comparative PCR. Comparative PCR was carried out in a Perkin-Elmer GeneAmp 9600 PCR system or, alternatively, in a peltier thermal cycler PTC-100 from M. J. Research, Inc. as described elsewhere (16). In brief, we prepared a master DNA solution of 5 ng/μl from blood DNA and different tumor DNA samples as determined by photometric measurement. Then we used this master DNA solution to generate three different dilutions (2, 1, and 0.5 ng/μl) of each DNA sample as determined by PCR. By using the three different DNA solutions, we monitored the linear range of amplification during the PCR reaction. To compare the gene copy number, three different concentrations of tumor and blood DNA were analyzed by PCR. Using the MUC primers and specific primers for clone 9-1, the concentrations of tumor DNA were adjusted to yield PCR products of equal intensity in tumor and blood DNA. PCR products were separated using a 2% agarose gel and stained with Sybr Green I (FMC Bioproducts). Subsequent to calibration, the amplification status of a chosen gene was analyzed by using gene-specific primers. The total volume of each PCR reaction was 50 μl with 0.5 μM of each primer, 200 μM deoxynucleotide triphosphates, and 2.5 units of Taq DNA polymerase (Pharmacia) in PCR buffer [500 mM KCl and 100 mM Tris-HCl (pH 9)]. The $MgCl_2$ concentration was 0.75 mM for the *MME* gene; 1.5 mM for the *MUC2* gene, the *KNG* gene, and the *APOD* gene; 2 mM for the *SI* gene; 2.5 mM for the *SLC2A2* gene; 3 mM for the *BCHE* gene; and 3.5 mM for clone 9-1. Primers for amplification of DNA from paraffin-embedded tissue were selected by the primer 0.5 program to generate PCR products of a maximal 173 bp in length (17, 18). Primer sequences are listed in Table 1. Amplification was carried out with 1-min denaturation at 94°C, 45-s annealing at 58°C, and 45-s extension at 72°C. The initial denaturation was for 5 min at 94°C, and the final extension was for 10 min at 72°C. The number of cycles was 26 for the *MUC* gene, 28 for amplification of the *SI* gene, 27 for the *BCHE* and *SLC2A2* genes, 30 for the *KNG* and *APOD* genes, 25 for the *MME* gene, 26 for the *HRG* gene, 24 for the *IL12A* gene, and 26 for clone 9-1 (19).

Southern Blot Analysis. For Southern blotting, we used 5 μg of genomic DNA as determined by photometric measurement. The DNA was restricted with *Eco*RI, separated on a 0.8% agarose gel, and transferred onto a nylon membrane in 0.4 M NaOH. A 300-bp fragment of the *BCHE* gene, a 450-bp fragment of the *SLC2A2* gene, and a 950-bp fragment of clone 8-6 were used as probes. These probes were labeled with ^{32}P using a random primer labeling kit (Boehringer Mannheim) and hybridized to the membrane overnight at 65°C. After washing the membrane three times at 65°C in 1% SDS and phosphate buffer (pH 7.2) at concentrations of 450, 250, and 150 mM, respectively, signals were identified by autoradiography using an intensifying screen (20).

RCP. RCP was conducted essentially as described previously (15). DNA was isolated from tumor tissues according to standard protocols and from paraffin-embedded tissue according to the QiaAmp Tissue kit (20). In brief, 1 μg of tumor DNA was labeled with biotin-16-dUTP by nick translation for 105 min, and 200 ng of labeled tumor DNA were combined with 20 μg of *Cot*1

Received 11/18/96; accepted 4/3/97.

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[1] Supported by Grant 10-1101-Me 2 from the Deutsche Krebshilfe and Grant TS 117/3/96 from the Heinrich Dietz-Stiftung. N. B. was a recipient of a grant from the Landesgraduiertenförderung.

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[3] The abbreviation used is: RCP, reverse chromosome painting.

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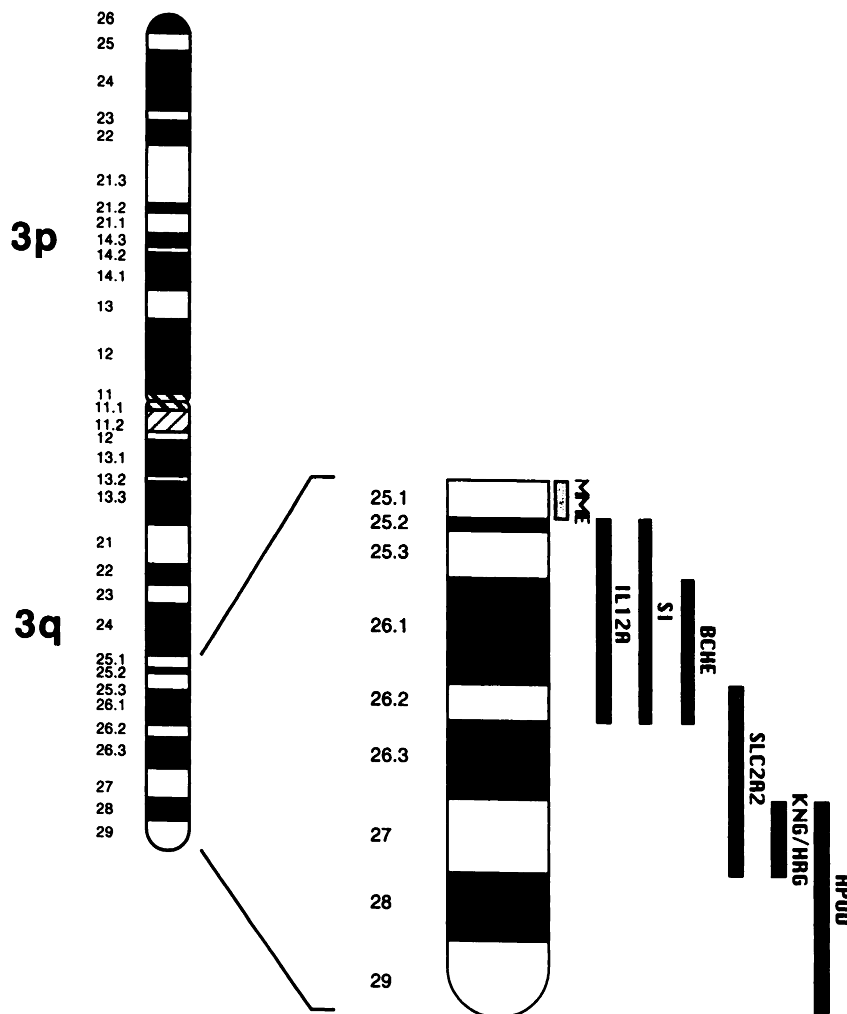

Fig. 1. Ideogram of the region on chromosome 3 analyzed for amplification. The location of the genes is given according to the Human Genome Database and to Leach *et al.* (29), respectively. The genes are referred to in Table 1.

DNA in a total hybridization volume of 2.5 μl. The cot value is defined as the product of the molar concentration of nucleotide residues and the time of renaturation. The probes were denatured at 75°C for 5 min, reannealed with *Cot1* DNA at 37°C for 10–20 min, and hybridized to normal metaphase chromosomes at 37°C overnight. After washes in a 50% formamide solution at 45°C and in 0.1× SSC at 60°C, biotinylated probes were detected using avidin conjugated to FITC. Counterstaining was by 4,6-diamino-2-phenyl-indol, and slides were mounted in fluorescence antifading buffer. Fluorescence signals were visualized in an Olympus microscope, captured by image integration with a Photometrics camera (CE 200A camera), and analyzed and documented with program ISIS3, version 1.53, of MetaSystems.

Table 1 *Genes used for amplification analysis*

The gene product, the gene symbol, the cytogenetic location of the genes, and the corresponding primer pairs used for comparative PCR are listed.

Gene product	Gene symbol	Cytogenetic location of the gene	Sequence of primers for comparative PCR (5′–3′ direction)
Membran-metallo-endopeptidase	*MME*	3q25.1	GGAGGGCTCTGGAAGTCAC ACAGCAGAATGGCAAATTCC
Interleukin 12A	*IL12A*	3q25.2–q26.2	CATGCTTTCAGAATTCGGGC CCTTCAGGAATGGATATTTTCCC
Sucrase-isomaltase	*SI*	3q25.2.q26.2	AGTATCCAGATGGTATGCAAGG CTTACCTGAGAAATTGTGGGC
Butyrylcholinesterase	*BCHE*	3q26.1–q26.2	ACAGCCACCTCTTGGTAGAC TTCCACATCTCTGATCCATG
Solute carrier family 2	*SLC2A2*	3q26.2–q27	AGCCTGTGGAGCCTGTAAG CACCAATGTCATATCCAAACTG
Kininogen	*KNG*	3q27	CCTGTGCATGAGCTTCTTAGG TTTCCTCGGACTGTGATTCC
Histidine-rich glycoprotein	*HRG*	3q27	GTGATTCCTTTGAAGAGGAAAATGAAT GCCCAAGCAGTAACAGAGACTCTA
Apolipoprotein D	*APOD*	3q27–qter	GGCACCAACAGTGCTAGAAAG CACAGCAGGTCAGCAACAAG
Mucin 2	*MUC*	11p15.5	CATTCTCAACGACAACCCCT GCAAGAGATGTTAGCTGCCC
Clone 9-1[a]	*9-1*	14q	TCCTATCTGCTTCTACCATCCC GCTTTATTGCCCTTGTCTATGC

[a] Gene product unknown.

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RESULTS

Genomic DNA was extracted from 16 tumor samples, including 5 biopsies and 11 paraffin-embedded tissues, and processed for RCP. In 3 of 16 cases, RCP showed strong hybridization signals on the entire long arm of chromosome 3. In three additional cases, the hybridization signals indicated various amplifications on 3q, all of which include chromosome band 3q26. Specifically, the latter amplifications map at 3q21–qter and 3q25.1–q26.2. These results were consistent with our previous observation of frequent amplification events on chromosome 3q, specifically, the involvement of chromosome band 3q26 (15). In total, the present and our previous RCP studies indicate amplifications including band 3q26 in 9 of 25 cases.

To determine whether known genes are involved in the amplification event, we used comparative PCR using primer pairs for eight genes assigned to 3q25.1–qter. The genes tested for amplification include *MME*, *IL12A*, *SI*, *BCHE*, *SLC2A2*, *KNG*, *HRG*, and *APOD* (Fig. 1 and Table 1). The gene *MUC*, which maps at 11p15.5, and clone 9-1, located at 14q, were used for calibration purposes. Comparative PCR was performed on tumor samples L10 and L26, which were shown to contain an amplification unit at 3q26 by using RCP. Whereas the genes *IL12A* and *APOD* were not amplified in either of the tumor samples, the genes *MME*, *KNG*, and *HRG* were amplified in one of the L10 or L26 tumors, respectively. The genes *SI*, *BCHE*, and *SLC2A2* were found to be amplified in both tumors, indicating a localization closer to the center of the amplification unit. Three examples for amplification analysis by comparative PCR are shown in Fig. 2. The results of the PCR experiments, which are consistent with our RCP data, are summarized in Table 2. To corroborate the PCR data, Southern blot analysis was performed for tumor L10, which yielded sufficient DNA to use both methods. As shown in Fig. 3, Southern blot analysis confirmed the amplification of *BCHE* and *SLC2A2* in tumor L10.

To determine the frequency of the gene amplifications, additional tumor samples were analyzed for amplification of the genes *SI*, *BCHE*, and *SLC2A2*. DNA was isolated from 34 paraffin-embedded tissues of squamous cell lung carcinomas. In 15 cases, the average size of the DNA fragments was sufficient for comparative PCR analysis. In the remaining 19 cases, the DNA fragments were only between 500 and 1500 bp in length. The DNA of the remaining cases could not have been efficiently amplified by PCR, although PCR primers were at a maximal distance of 173 bp. Of the 15 squamous cell lung carcinomas, 6 showed amplification of at least 1 of 3 genes (*SI*, *BCHE*, and *SLC2A2*). Although the gene *SI* was amplified in only a single case (P1), the genes *BCHE* and *SLC2A2* were amplified in five cases each. As summarized in Table 3, the genes *BCHE* and *SLC2A2* were coamplified in four tumors. In total, 40% of the squamous cell lung carcinomas showed an amplification of at least one gene at 3q26.

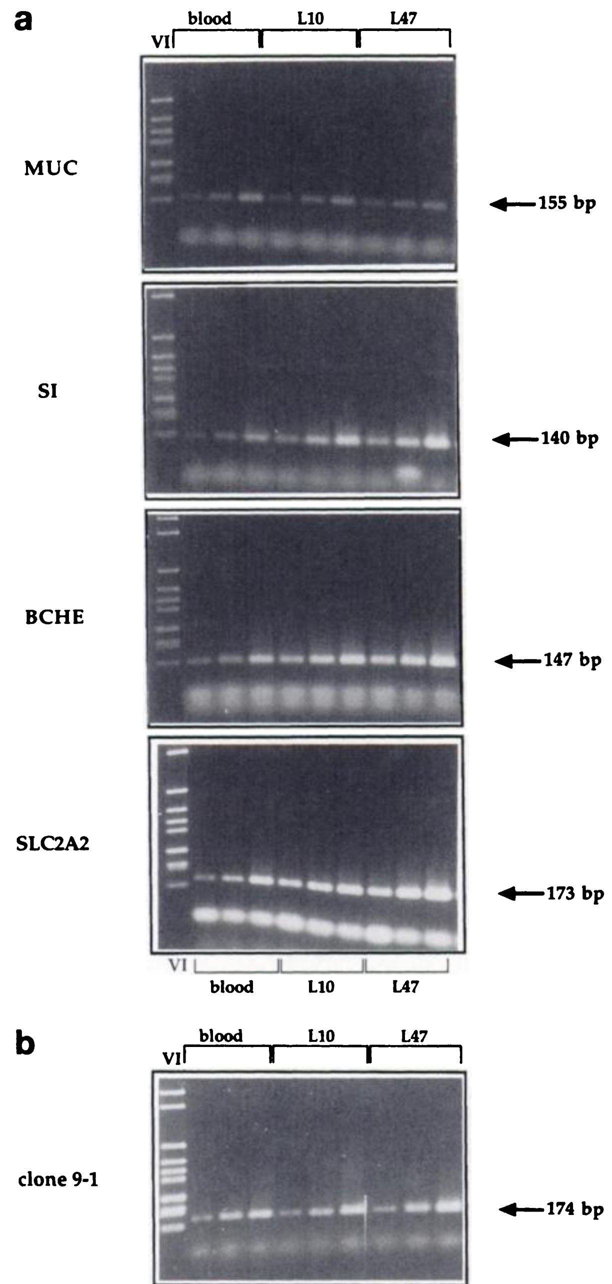

Fig. 2. Representative analysis of gene amplification by comparative PCR. DNA from blood and tumors L10 and L47 was amplified by PCR, and the PCR products were separated by gel electrophoresis and stained by Sybr Green I. *Lane 1*, DNA marker; *Lanes 2–4*, three dilutions of blood DNA; *Lanes 5–7*, three dilutions of DNA from tumor L10; *Lanes 8–10*, three dilutions of DNA from tumor L47. Tumor L47 and L10 were shown to harbor an amplification on 3q by RCP. *a*, PCR was performed with primer pairs specific for the genes *SI*, *BCHE*, *SLC2A2*, and *MUC*. The gene *MUC* was used for calibration purposes. The strong signal intensity of all three tumor DNA dilutions reflects an increased copy number of the genes for *SI*, *BCHE*, and *SLC2A2* for both tumors (L10 and L47). *b*, PCR was performed with a primer pair specific for clone 9-1, which maps on chromosome 14q. This chromosome region is usually not involved in gene amplifications in squamous cell lung carcinoma.

DISCUSSION

Our RCP data indicate that chromosome band 3q26 is involved in amplification events in more than 35% of squamous cell lung carcinoma. Although RCP is a powerful tool for the detection of amplified domains, this approach is fraught with several limitations. Specifically, small and low- level amplification units are less likely to be detectable by RCP (21). The likelihood of identifying an amplification unit is further reduced by the use of biopsy material. The presence of normal cells such as blood cells in the tumor biopsy complicates the search for amplified domains by RCP. To circumvent these limitations, we analyzed amplifications not only by RCP but also by using gene-specific PCR primers. In addition, the analysis was not restricted to biopsy material but was mostly performed on paraffin-embedded tissues.

The major drawback of the paraffin-embedded tissue was the reduced quality of the DNA (22). Approximately 50% of the paraffin samples yielded DNA that was of sufficient size for PCR analysis. To increase the amplification efficiency, we chose primer pairs with a maximal distance of 173 bp. Furthermore, PCR primers were chosen to obtain PCR products of similar sizes for the *MUC* gene and for the

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genes that were tested for amplification. As expected, the analysis of single genes by PCR detected a slightly higher frequency of amplifications (40%) than the analysis by RCP (36%). An amplification level of approximately 40% has been observed in few human tumors including glioblastoma, with the *EGFR* gene amplified in 30–50% of this tumor (23). Highly amplified genes lend themselves as molecular prognostic markers. Amplified *NMYC* and *NEU* have been proposed as prognostic parameters in neuroblastoma and in breast carcinoma, respectively (24, 25). As of yet, comparable levels of amplification have not been observed in lung tumors. Both *BCHE* and *SLC2A2* genes are possible candidates for molecular prognostic markers in squamous cell carcinoma of the lung. Their functional role in the tumorigenesis of squamous cell carcinoma of the lung has to be determined in additional studies. Possibly, *BCHE* and *SLC2A2* indicate nearby genes that are important in the pathogenesis of squamous cell lung carcinoma.

Abnormal expression and amplification of the *BCHE* gene have been reported for several kinds of tumors, including leukemia, brain cancer, and ovarian cancer (26). The true physiological function of serum cholinesterase has not been identified (27). *SLC2A2* plays a crucial role in the regulation of systemic blood glucose level. Although there are numerous studies elucidating the role of *SLC2A2* in impaired glucose metabolism, there are limited data indicating a specific involvement of *SLC2A2* in the genesis of human cancers. A high *SLC2A2* expression observed in head and neck tumors was not associated with amplifications or DNA rearrangements (28).

Table 2 *Amplification of genes at 3q25–qter in the squamous cell lung carcinomas L10 and L26 as determined by comparative PCR*

Amplification is indicated by +, absence of amplification is indicated by −.

Gene	Sample	
	L10	L26
MME	−	+
IL12A	−	−
SI	+	+
BCHE	+	+
SLC2A2	+	+
KNG	+	−
HRG	+	−
APOD	−	−
MUC	−	−

Table 3 *Amplification of the genes SI, BCHE, and SLC2A2 in squamous cell lung carcinomas as determined by comparative PCR*

DNA was isolated from paraffin-embedded tissues. Amplification is indicated by +, absence of amplification is indicated by −.

Sample	Gene		
	SI	*BCHE*	*SLC2A2*
P1	+	−	+
P2	−	−	−
P3	−	−	−
P6	−	−	−
P11	−	+	+
P12	−	−	−
P14	−	+	+
P17	−	−	−
P18	−	−	−
P19	−	−	−
P21	−	+	+
P22	−	+	−
P27	−	+	+
P28	−	−	−
P33	−	−	−

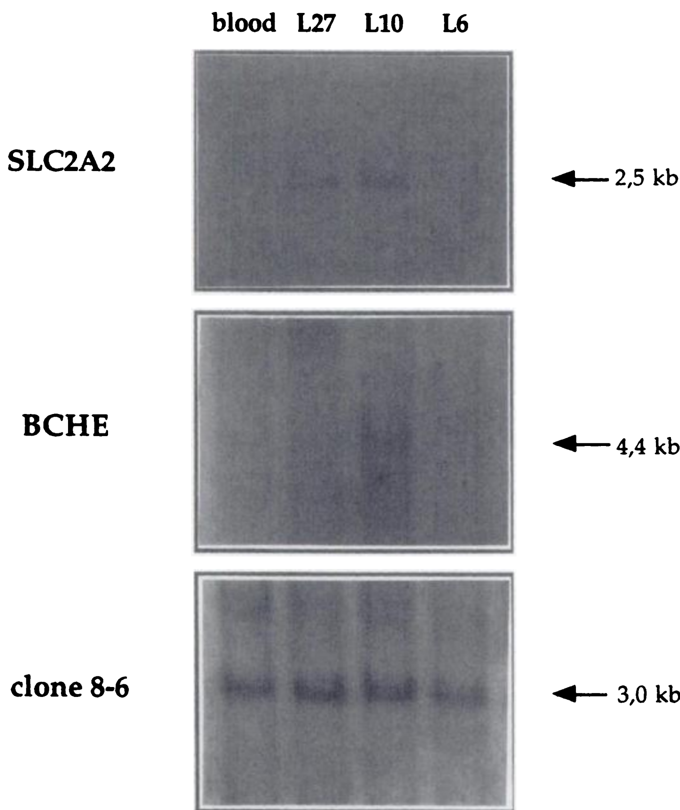

Fig. 3. Demonstration of gene amplification by Southern blot analysis. DNA from blood and tumors (L27, L10, and L6) was restricted by *Eco*RI and hybridized with ^{32}P-labeled probes of the *BCHE* and *SLC2A2* genes. Probe clone 8-6 was used for calibration purposes.

Additional studies are needed to elucidate the molecular mechanisms that contribute to the development of non-small cell carcinoma of the lung and that involve the amplified genes *BCHE* and *SLC2A2*. To investigate the role of these genes in squamous cell lung carcinoma, we established an expression library from tumor L10, which was known to carry an amplified domain at 3q26. The library was screened using several PCR primers, including primer pairs for the *BCHE* and the *MME* gene. The preliminary results were consistent with our amplification analysis. Although PCR failed to identify a *MME* cDNA, there was a strong PCR product with the *BCHE*-specific primers. As summarized in Table 2, the *BCHE* gene was clearly amplified in tumor L10, whereas no amplification has been found for the *MME* gene. Further expression analysis has not been performed due to the lack of sufficient tumor material.

In summary, our study demonstrates DNA amplification at 3q26 in approximately 40% of squamous cell lung carcinoma, with the genes *BCHE* and *SLC2A2* as the genes most frequently amplified. Our preliminary expression studies demonstrate the expression of the *BCHE* gene in a tumor sample that carries the amplified domain. The high frequency of amplification warrants further analysis of the region 3q26, specifically, of the *BCHE* and *SLC2A2* genes and their involvement in the pathology of squamous cell carcinoma of the lung.

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