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Research Paper

Amplification of Chromosome 8 Genes in Lung Cancer

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Abstract

Chromosomal alterations are frequent events in lung carcinogenesis and usually display regions of focal amplification containing several overexpressed oncogenes. Although gains and losses of chromosomal loci have been reported copy number changes of the individual genes have not been analyzed in lung cancer. In this study 22 genes were analyzed by MLPA in tumors and matched normal tissue samples from 82 patients with non-small cell lung cancer. Gene amplifications were observed in 84% of the samples. Chromosome 8 was found to harbor the most frequent copy number alterations. The most frequently amplified genes were ZNF703, PRDM14 and MYC on chromosome 8 and the BIRC5 gene on chromosome 17. The frequency of deletions were much lower and the most frequently deleted gene was ADAM9. Amplification of the ZNF703, PRDM14 and MYC genes were highly correlated suggesting that the genes displaying high copy number changes on chromosome 8 collaborate during lung carcinogenesis.

Key words: Oncogenes, copy number changes, lung cancer

INTRODUCTION

Lung cancer is a major cause of cancer deaths throughout the world and is classified into two categories. Small cell lung cancer (SCLC) constitutes 15-20% of the lung cancers while non-small cell lung cancer (NSCLC) accounts for the remaining group. NSCLC includes squamous cell carcinoma, adenocarcinoma, adenosquamous cell carcinoma and large cell carcinoma [1, 2]. The development and progression of lung carcinoma is a long process that involves multiple cancer-related genes, as well as environmental and host genetic factors. Advances in molecular biology have shown that lung cancer is caused by the complex interaction of genetic and environmental factors and by accumulation of multiple genetic alterations such as amplifications, deletions, mutations and epigenetic events [2-4]. Although smoking is the main cause of lung cancer approximately 10% of cases are never smokers [5]. Despite advances in the therapeutic modalities the overall 5-year survival rate is still less than 15% for NSCLC and even lower for SCLC [6].

Tyrosine kinase inhibitors (TKIs) are used commonly to treat patients harboring activating mutations in the tyrosine kinase domain of the epidermal growth factor receptor gene. Patients who respond to TKI treatment are usually those who have never smoked and have generally adenocarcinomas [6-8]. Furthermore, during inhibitor therapy these patients develop resistance to treatment [9-10]. Another group of patients are individuals carrying K-Ras mutations with a history of smoking. These patients are also associated with resistance to EGFR-TKIs [11, 12]. Therefore, patient survival has not improved significantly and many unanswered questions about the optimal use of TKIs still remain.

To achieve a better and more stable response combined analysis of the relevant pathways which are involved in the mechanism of killing the tumor cells

by chemotherapy is necessary. Identification of key molecular events during lung carcinogenesis and tumor progression will facilitate characterization of new therapeutic targets and development of new treatment strategies. Therefore, numerous studies aim to identify new molecular biomarkers which may predict a better clinical outcome for the patients.

Like in other solid tumors, chromosomal aberrations represent critical molecular events in the pathogenesis of lung cancer and recurrent genomic alterations have been reported in NSCLC [13]. The loci of recurrent aberrations frequently display regions of focal amplification in a wide range of tumors. Most of these regions harbor several oncogenes and the number of the amplified genes as well as the level of amplification may differ depending on the type of the tumor [13, 14]. Genes overexpressed within these amplicons are attractive targets for therapy. Frequently, two or more of these amplicons are present in a given tumor, suggesting that genes in the two amplified regions may collaborate in the development of the transformed phenotype [15, 16].

In contrast to other high throughput analysis methods, multiplex ligation-dependent probe amplification (MLPA) is an inexpensive and reliable method for the simultaneous analysis of a high number of different loci [17]. Although gains of partial or whole chromosomal arms on 1q, 3q, 5p, 8p and 11q have been reported in NSCLC copy number changes of the genes localized in these loci have not been analyzed in lung cancer [18-20]. In this study in order to understand the molecular background and to explore new targets for prevention and therapy, we investigated gene copy number changes in 22 genes in a group of 82 NSCLC tumors and matched non-cancerous tissue samples using MLPA.

MATERIALS AND METHODS

Tumor tissue and the corresponding normal lung tissue samples were obtained from 82 patients undergoing surgery at the Istanbul University Cerrahpasa Medical Faculty, Department of Chest Surgery. The patients taken into the study had not undergone any previous treatment and were admitted to the hospital for removal of the tumor as primary treatment. DNA was obtained by digestion of the tissues using a DNA isolation kit (Roche Diagnostics DNA Isolation Kit-Mannheim, Germany) according to the manufacturer's instructions.

Copy number variations of 22 genes were analyzed by MLPA in 82 patients with NSCLC. MLPA analysis was performed by using 100 ng of genomic DNA and the SALSA MLPA P078-C1 Probemix kit (MRC-Holland, Amsterdam, Netherlands) according to the manufacturer's instructions. The list of the genes and the probes used in the study are shown in Table 1. The samples were analyzed in duplicate using the ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Raw data were normalized using the GeneMapper program (Applied Biosystems, Foster City, CA, USA). Relative peak areas of the amplification products were evaluated using the Coffalyzer MLPA analysis software (MRC-Holland, Amsterdam, the Netherlands) and compared with results obtained from the healthy tissue specimens to calculate gene copy numbers. For genes analyzed with multiple probes, the mean of all the peak values for this gene was calculated. A mean value below 0.75 was defined as loss, values between 0.75 and 1.25 were defined as normal, between 1.25 and 1.65 as heterozygote duplications, between 1.65 and 2.3 as triplications and between 0.25 and 0.75 as heterozygote deletions.

The study was approved by the Ethics Committee of Istanbul University, Cerrahpasa Medical Faculty and written informed consent was obtained from all patients enrolled in the study.

Statistical analysis was performed using the SPSS 21 for Windows (IBM Corp. Released version 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.). We used the χ^2 (2-tailed) test to analyze the association between gene copy number variations and clinicopathological characteristics and the Spearman's Rho test to evaluate the correlation between the most frequently amplified genes. $p<0.05$ was considered statistically significant.

Table 1. List of the genes analyzed by MLPA in the tumor and matched normal tissue samples.

GENE	Chromosome	Description
ESR1	6q25	Transcription factor
EGFR	7p11	Receptor tyrosine kinase in signaling
ZNF703	8p11	Transcription factor in migration and proliferation
FGFR1	8p11	Receptor tyrosine kinase in signaling
ADAM9	8p11	Metalloproteinase in protein metabolism
IKBKB	8p11	Serine threonine kinase in signaling
PRDM14	8q13	Transcription regulatory protein
MTDH	8q22	Transcription co-activator
MYC	8q24	Transcription factor in apoptosis and proliferation
CCND1	11q13	Cell cycle control protein in signaling
C11ORF30	11q13	Transcription regulatory protein
CDH1	16q22	Adhesion molecule in signaling
CPD	17q11	Carboxylpeptidase in protein metabolism
MED1	17q12	Component of mediator complex
ERBB2	17q12	Receptor tyrosine kinase in signaling
CDC6	17q21	Cell cycle control protein in signaling
TOP2A	17q21	DNA topoisomerase protein
MAPT	17q21	Microtubule assembly promoter
PPM1D	17q23	Serine threonine phosphatase
BIRC5	17q25	Transcription factor in apoptosis and proliferation
CCNE1	19q12	Cell cycle control protein in signaling
AURKA	20q13	Serine threonine kinase in signaling

RESULTS

To investigate whether there is a change in cancer-associated gene copy numbers in tumors from patients with non-small cell lung cancer, we performed MLPA analysis in 82 tumors and matched normal tissue samples. Amplification of genetic material was found in 69 of 82 (84.14%) of the NSCLC tissues analyzed by the MLPA assay, while the remainder of the tumors did not display amplification among the tested 22 genes (Table 2). Losses were observed in 21.9% of the patients.

Chromosome 8 harbored the most frequent alterations. We used 7 and 5 probes hybridizing to the short and long arms of chromosome 8, respectively. Altogether, fifty five samples (67%) showed amplification of the ZNF703, PRDM14 and/or MYC genes. The most frequently amplified gene on the short arm of chromosome 8 was ZNF703 with a frequency of 46.3%. In 14 (17%) of these cases ZNF703 was the only amplified gene. PRDM14 and MYC genes on the long arm were amplified in 41.5% and 32.9% of the patients, respectively. Exclusive amplifications of the PRDM14 and MYC genes were present in 9 (10.9%) and 4 (4.9%) out of 55 cases, respectively. In the remaining 28 (34.1%) samples concurrent amplification of all or any two of these genes were observed at the same time (Table 3). 73.9%, 70.4% and 67% of these amplifications were duplications and 26.1%, 29.6% and 33.6% were triplications of the ZNF703, PRDM14 and MYC genes, respectively.

Table 2. CNV analysis of the 22 genes in NSCLC tumor tissue.

Genes	No change	Duplication	Triplication	# of amplified samples	Heterozygote Deletion	Homozygote Deletion	Amplification %
ESR1	77	4	0	4	1	0	4.9
EGFR	67	7	8	15	0	0	18.3
ZNF703	43	28	10	38	1	0	**46.3**
FGFR1	68	6	3	9	5	0	11.1
ADAM9	69	3	4	7	6	0	8.5
IKBKB	73	7	2	9	0	0	11.1
PRDM14	58	24	10	34	0	0	**41.5**
MTDH	64	12	4	16	2	0	19.5
MYC	55	18	9	27	0	0	**32.9**
CCND1	72	2	8	10	0	0	12.2
C11ORF30	68	7	3	10	4	0	12.2
CDH1	70	7	4	11	1	0	13.4
CPD	69	6	2	8	5	0	9.8
MED1	62	9	5	14	4	2	17.1
ERBB2	71	6	5	11	0	0	13.4
CDC6	68	9	4	13	1	0	15.9
TOP2A	74	6	1	7	1	0	8.5
MAPT	75	3	2	5	2	0	6.1
PPM1D	62	12	4	16	4	0	19.5
BIRC5	55	25	2	27	0	0	**32.9**
CCNE1	64	13	5	18	0	0	22.1
AURKA	73	9	0	9	0	0	11.1

Table 3: Frequency and coamplification of the most frequently amplified genes.

GENES	n=82 (%)
ZNF703-PRDM14-MYC	12 (14.63)
ZNF703-PRDM14	9 (10.97)
ZNF703-MYC	4 (4.87)
PRDM14-MYC	3 (3.65)
ZNF703	14 (17.07)
PRDM14	9 (10.97)
MYC	4 (4.87)

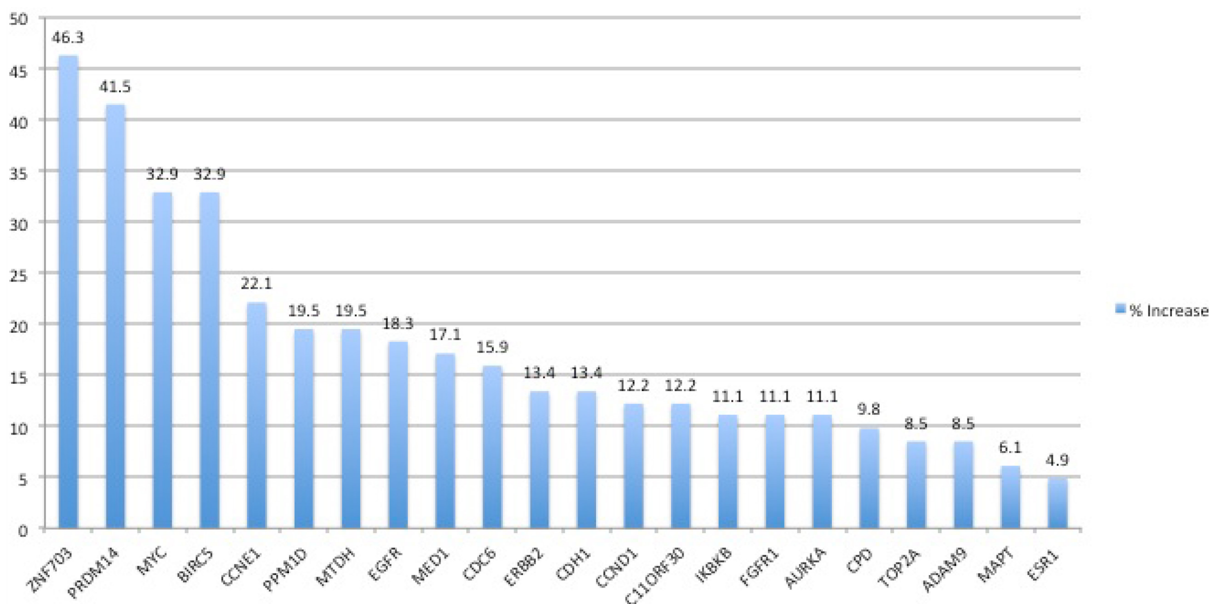

Figure 1: The frequency of gene amplifications in NSCLC tumors as determined by MLPA

Table 4. Distribution of CNVs in association with clinicopathological characteristics.

		CNV							
n=82		1 gene	2-3 genes	4-6 genes	≥ 7 genes	No change	Decreased	Total	p
Pathology	Adeno Ca	5 (15.6)	10 (31.3)	5 (15.6)	5 (15.6)	6 (18.8)	1 (3.1)	32	
	SCC	7 (20.0)	7 (20.0)	4 (11.4)	11 (31.4)	6 (17.1)	0 (0)	35	0.815
	n/a	4 (26.7)	4 (26.7)	2 (13.3)	4 (26.7)	1 (6.7)	0 (0)	15	
Stage	Stage 1A-2A	3 (12.0)	6 (24.0)	3 (12.0)	8 (32,0)	5 (20.0)	0 (0)	25	
	Stage 2B-4	1 (6.3)	2 (12.5)	3 (18.8)	6 (37.5)	4 (25.0)	0 (0)	16	0.828
	n/a	12 (29.3)	13 (31.7)	5 (12.2)	6 (14.6)	4 (9.8)	1 (2.4)	41	
Gender	Male	13 (18.6)	16 (22.9)	10 (14.3)	17 (24.3)	13 (18.6)	1 (1.4)	70	
	Female	2 (22.2)	4 (44.4)	0 (0)	3 (33.3)	0 (0)	0 (0)	9	0.321
	n/a	1 (33.3)	1 (33.3)	1 (33.3)	0 (0)	0 (0)	0 (0)	3	
Age	< 50 years	3 (30.0)	2 (20.0)	1 (10.0)	3 (30.0)	0 (0)	1 (0)	10	
	≥ 50 years	12 (17.6)	17 (25.0)	9 (13.2)	17(25.0)	13 (19.1)	0 (0)	68	0.560
	n/a	1 (25.0)	2 (50.0)	1 (25.0)	0 (0)	0 (0)	0 (0)	4	
Smoking Status	30 packs/year	6 (19.4)	6 (19.4)	4 (12.9)	7 (22.6)	8 (25.8)	0 (0)	31	
	31-60 packs/year	6 (21.4)	6 (21.4)	3 (10.7)	9 (32.1)	4 (14.3)	0 (0)	28	
	>60 packs year	1 (20.0)	2 (40.0)	1 (20.0)	0 (0)	1 (20.0)	0(0)	5	0.854
	n/a	3 (16.7)	7 (38.9)	3 (16.7)	4 (22.2)	0 (0)	1 (5.6)	18	

On the other hand, BIRC5 was the most frequently amplified gene on chromosome 17. There were 15 probes available hybridizing with 8 genes on the long arm of chromosome 17. Amplification of the BIRC5 gene was observed in 32.9% of the samples with the same frequency as for the MYC gene. The remaining genes analyzed in this study were amplified with frequencies varying between 4.9% and 22.1%. In 20 (24.3%) samples amplification of 7 or more genes were observed.

The mean copy numbers in the amplified samples were 1.31±0.36 for ZNF703, 1.27±0.36 for PRDM14, 1.29±0.46 for MYC and 1.23±0.25 for the BIRC5 genes. When we analyzed the correlation between the clinicopathological characteristics of the patients and gene amplification status, no association was observed with any clinical parameter (Table 4).

The frequency of the deletions was considerably low (Table 2). The most frequently deleted gene was ADAM9 and was observed in 6 of 82 (7.3%) of the tumor samples while deletion of FGFR1 was observed in 5 (6.1%) tumors. The only gene showing a homozygous deletion was MED1 in 2 (2.4%) of the patients.

Finally, we applied multivariate analysis for the 4 most frequently altered genes to evaluate whether variations in these genes affect each other. A significant positive association was observed between the PRDM14 and ZNF703 (p=0.00), MYC (p=0.01) and BIRC5 (p=0.01) genes. A positive correlation was also present between the ZNF703 and MYC genes (p=0.04). These data indicate that copy number variations in the genes located on chromosome 8 may affect other genes located on the same chromosome.

DISCUSSION

Molecular cytogenetic methods such as fluorescence *in situ* hybridization (FISH), comparative genomic hybridization (CGH), and spectral karyotyping (SKY) have greatly improved our abilities to detect genome-wide copy number alterations in solid tumors [21, 22]. Genome-wide and massively parallel sequencing analyses have shown the presence of recurrent abnormalities in different regions including amplifications in a broad range of epithelial cancers [16]. Therefore, to develop more accurate diagnostic and therapeutic strategies, several investigators have focused on the identification of chromosomal aberrations associated with NSCLC using CGH or FISH [13, 14, 23, 24]. Results of these investigations have shown that recurrent genomic alterations such as gains of partial or whole chromosomal arms on several chromosomes along with losses of others are present in NSCLC. However, the resolution of conventional CGH is not sufficient for the precise identification of the submicroscopic molecular changes on the gene level [21]. In recent years a new method of high-throughput molecular analysis, the multiplex ligation-dependent probe amplification (MLPA) has been introduced. However, the potential utility of this approach has not been evaluated for the analysis of gene copy numbers in lung cancer yet. There is only a single report in the literature using MLPA for methylation analysis in lung tumors [25]. Since it requires only small quantities of DNA and is much more sensitive and reliable than cytogenetic analysis we performed MLPA analysis to investigate the copy number alterations in matched tumor tissues [17].

Among the 8 chromosomes which were investigated in this study chromosome 8 was the most frequently amplified chromosome. In previous studies numerical chromosomal aberrations have been reported for chromosome 8. However, the MYC oncogene has been reported as the most frequently amplified gene in various tumor types [26]. On the other hand, Kubokura et al. reported that chromosome 8

copy number alterations were not associated with MYC amplification in NSCLC [27]. In this study we analyzed 7 different genes on the long and short arms of chromosome 8. Although one of the amplified genes on the long arm of chromosome 8 was MYC it was not the primarily amplified gene on chromosome 8. The most frequently amplified genes were the ZNF703 (zinc finger protein 703) gene on the short arm, and PRDM14 on the long arm of chromosome 8.

ZNF703 is a member of the NET/NIZ family of transcription factors and has been identified recently as a novel oncogene in human breast cancer [16, 28, 29]. It has been characterized as the genetic driver of the A1 amplicon on chromosome 8 and has been implicated in different properties of the cancer cells including renewal, proliferation and invasion [30]. Overexpression of ZNF703 in lung cancer is in accordance with data observed in breast and gastric tumors [30-32]. Amplification of ZNF703 has been found to be second only to erbB2 and CCND1 genes in breast cancer and it has been shown that ZNF703 overexpression increases genome instability and contributes to tumor aggressiveness in breast cancer [16, 33]. More recently, enhanced ZNF703 expression has been correlated with repression of E-cadherin and increased lung metastasis rates in breast cancer [34]. These findings indicate that ZNF703 may function as an oncogene in different types of cancer. It remains to be determined how the expression and function of the ZNF703 protein is regulated.

The second most frequently amplified gene in our series was PRDM14 which is located on 8q13. PRDM14 is a member of the PRDM family of transcriptional regulators and controls pluripotency and epigenetic reprogramming by repressing/activating relevant genes through a variety of different mechanisms [35, 36]. PRDM14 is one of the key transcriptional regulators of primordial germ cell specification and over-expression of PRDM14 has been reported in different cancers [37-40]. Recently, the PRDM14 gene has been identified as a susceptibility locus for cancer [41]. Its amplification and over-expression has been associated with a more aggressive phenotype and reduced sensitivity to chemotherapy in breast cancer [37]. There are only two studies in the literature investigating the expression rate of PRDM14 in NSCLC tumors by immunohistochemistry and western blotting reporting that PRDM14 is strongly expressed in NSCLC [42, 43]. The authors also report a correlation between PRDM14 expression levels and differentiation. In our study group the frequency of tumors with high PRDM14 expression was lower than reported by Liu et al. [42] but in line with Zhang et al. [43] who observed expression in 35 % of the tumors.

The remaining frequently amplified genes were the BIRC5 and MYC oncogenes in accordance with previous reports. Data from a recent study suggests that BIRC5 may exert its effect by modulating Arf6 expression [44]. There are many reports in the literature indicating overexpression of these two genes in NSCLC and other cancers [45-49]. However, the more interesting finding is the frequent co-amplification of the genes which are located on the same chromosome.

Some of the genes within the amplicons are oncogenes that drive the amplicon while others are passengers. In our study group none of the amplified genes are located on the same amplicon. However, we observed significant correlations between the amplified genes, suggesting a collaborative role of different amplicons in the tumorigenesis of NSCLC. Our data indicate that amplification of the multiple amplicons on chromosome 8 are an important and significant contributor to NSCLC pathogenesis since collaboration between oncogenes within the different regions of the same chromosome are observed. Contribution of the regions on chromosome 8 may provide a selective advantage for the transformed phenotype. Further studies will be required to identify the associated genes and their mutual interactions in order to elucidate their role in lung carcinogenesis.

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Competing Interests

The authors have declared that no competing interest exists.

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