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Original Paper

DNA Amplification on Chromosome 3q26.1–q26.3 in Squamous Cell Carcinoma of the Lung Detected by Reverse Chromosome Painting

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Multiple genetic lesions have been reported in small cell lung carcinoma (SCLC), while considerably less information is available on squamous cell carcinoma (SCC). We used reverse chromosome painting to screen a total of nine SCCs for DNA amplifications. In three of the nine SCCs, hybridisation signals were found at chromosome region 3q26.1–q26.3, which does not contain any known oncogene. In one of the three SCCs, there were additional hybridisation signals at 1q, 5p and 6p21.1. The high frequency of a consistent amplification (3q26.1–q26.3) in SCC strongly indicates a novel gene at 3q26.1–q26.3 that is important in the pathology of SCC. Copyright © 1996 Elsevier Science Ltd

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INTRODUCTION

DNA AMPLIFICATION has been reported for a wide spectrum of cancers and appears to contribute to the malignant phenotype [1, 2]. There is, however, no example of developmentally regulated gene amplification in mammals. In some instances, DNA amplification has been shown to be of prognostic significance, most notably amplification of the gene *NEU* in human breast cancer and the gene *NMYC* in neuroblastoma [3–6].

There is increasing evidence for gene amplification in non-small cell lung cancers (NSCLC) that account for 75–80% of all lung tumours [7, 8]. Double minutes (DMs) as cytogenetic manifestations of gene amplification were found in 77% of short-term cultures derived from NSCLC [7]. Southern blot analysis revealed amplification of several proto-oncogenes in NSCLC including *CMYC* and *KRAS2* [8–10]. There were also reports of amplifications of the topoisomerase II gene and the gene for parathyroid hormone-related protein (PTHRP) in NSCLC [8, 11]. Taken together, the emerging pattern of gene amplification in NSCLC appears to be rather complex, suggesting different amplified genes are involved in the pathogenesis of NSCLC. Cytogenetic analysis of NSCLC has consistently revealed an accumulation of multiple chromosome alterations, indicating several independent genetic events as first shown for colon cancer [12–14].

Although the high incidence of DMs in NSCLC emphasises the crucial role of DNA amplifications in this tumour type, there is virtually no information on the chromosomal origin of these extrachromosomal amplification units. The genes known to be amplified in NSCLC are likely to account only for a small percentage of the total number of amplification events. To identify and localise novel amplification units in NSCLC, reverse chromosome painting was employed. Labelled genomic DNA from tumour cells and unlabelled sonicated DNA from normal cells were hybridised as a probe against a metaphase spread with normal male karyotype [15, 16]. Amplified DNA sequences in tumour DNA were indicated by intense fluorescence hybridisation signals.

Reverse chromosome painting has successfully been applied to identify amplified domains in human tumour cells [17, 18]. Most recently, 22 different amplified domains have been reported in small cell lung carcinomas (SCLCs) using comparative genomic hybridisation (CGH) [19]. The aim of this study was to identify amplified domains as yet unknown in squamous cell carcinoma (SCC) using reverse chromosome painting.

MATERIALS AND METHODS

Isolation and labelling of tumour DNA

Tissue samples of SCC of the lung were frozen in liquid nitrogen immediately after biopsy and stored at −70°C. Gen-

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omic DNA was isolated from the tumour samples by standard methods [20]. In brief, after proteinase K digestion at 55°C, proteins were extracted with chloroform and DNA was precipitated with isopropanol. Tumour DNA was labelled with biotin-16-dUTP by nick translation (Nick Translation System, Gibco BRL, Germany).

Isolation and preparation of DNA for competition

High molecular weight DNA was prepared from lymphocytes of normal karyotype (46XX) according to standard protocols [20]. DNA was sonicated and fragments of 200–500 bp were found by agarose gel electrophoresis.

Metaphase chromosome spreads

Metaphase chromosomes were prepared from peripheral blood lymphocytes (46XY) according to standard methods [21] and suspensions were stored in methanol:acetic acid (3:1) at −20°C.

In situ *hybridisation*

Biotin-labelled tumour DNA (150–200 ng) was mixed with sonicated normal DNA at a ratio of 1:1 and combined with 40 μg COT-1 DNA in a total hybridisation volume of 2.5 μl. Alternatively, labelled tumour DNA was hybridised in the presence of a 100-fold excess of COT-1 DNA without competition by sonicated normal DNA. The probes were denatured at 75°C for 5 min, reannealed at 37°C for 10–20 min and hybridised to normal male metaphase chromosomes at 37°C overnight. Prior to hybridisation, slides were treated with RNase A for 1 h and pepsin for 10 min. After dehydration in ethanol, chromosomes were denatured in a 70% formamide solution at 80°C for 2 min and again dehydrated by three cold ethanol washes (70%, 80%, 96%).

Detection

Following 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 fluorescein isothiocyanate. One round of amplification was performed by using goat anti-avidin antibodies. Slides were counterstained with 4,6-diamino-2-phenyl-indol (DAPI) and mounted in fluorescence-antifading buffer. Fluorescence signals were visualised in a Zeiss microscope, captured by image integration with a Photometrics camera (CE 200A Camera), analysed and documented with the program ISIS3, version 1.53 of MetaSystems. To be counted as positive, hybridisation signals were required in at least 45 of the 50 metaphases analysed.

RESULTS

To detect DNA amplifications, a total of nine SCCs were analysed by reverse chromosome painting. Three specimens were histopathologically characterised as WHO (World Health Organisation) grade I and five specimens as WHO grade III tumours. Tumour DNA was labelled by biotin-16-dUTP mixed with COT-1 DNA and unlabelled sonicated blood DNA, and hybridised against metaphase spreads with normal male karyotype. Defined fluorescence hybridisation signals were found in three of the nine cases, indicating a high frequency of gene amplification in SCCs. There was no difference between hybridisations performed with and without unlabelled genomic DNA. In all three cases, an amplification event was localised within region 3q26.1–q26.3. In one of the three SCCs, there were additional hybridisation signals at 1q, 5p and 6p(21.1). Our results are strongly indicative of an amplified gene at 3q26.1–q26.3 which is likely to be of importance in the pathology of SCC. Figure 1 shows a representative reverse chromosome painting experiment demonstrating the hybridisation signal at 3q26.1–q26.3. To our knowledge, there are no oncogenes within chromosome region 3q26.1–q26.3, indicating a novel amplified gene residing within this chromosome region. The results of the reverse chromosome painting experiments are summarised in Table 1, including pathological staging of the tumours.

DISCUSSION

Our data demonstrate a specific involvement of a region on chromosome 3q in SCC. In previous studies, many genetic alterations have been associated with lung cancer, the majority with SCLC [22, 23]. In both SCLC and NSCLC, the most frequent chromosomal alterations are deviations of chromosome 3, with loss of the short arm as the most consistent finding [22, 24–26]. Previously, Ried and colleagues [19] reported a simultaneous over-representation of the long arm of chromosome 3 in primary SCLC. The loss of the short arm of chromosome 3, together with the over-representation of the

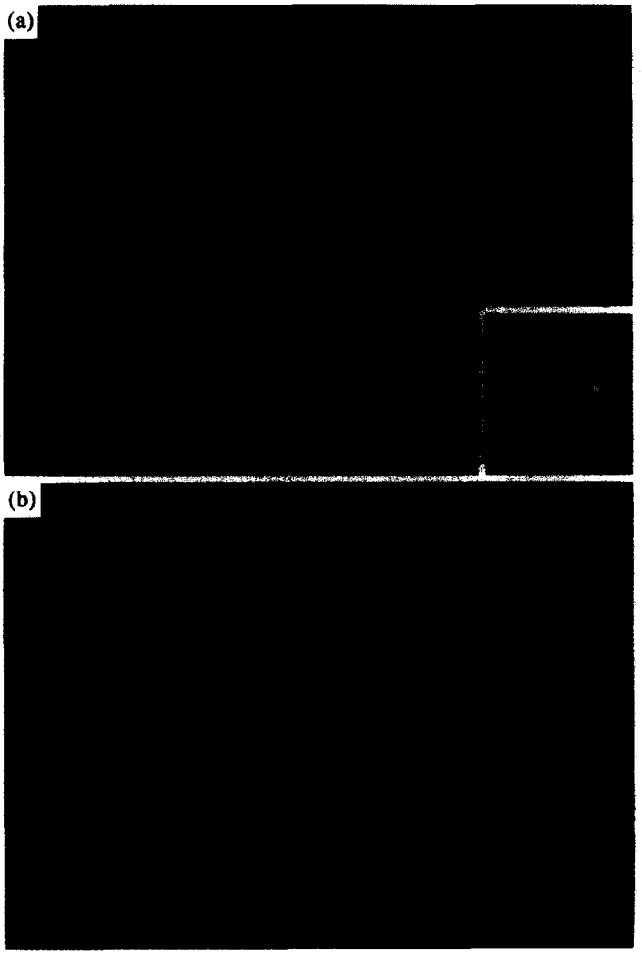

Figure 1. (a) Reverse chromosome painting using fluorescence-labelled DNA from tumour L26. Hybridisation signals were identified at 3q26.1–q26.3 (arrows). (b) Metaphase spread shown in (a), counterstained with 4,6-diamino-2-phenyl-indol. The inset to (a) depicts a second case of reverse chromosome painting analysis. Hybridisation signals at 3q26.1–q26.3 were identified using DNA from tumour L1.

Table 1. Clinicopathological features of nine patients with lung cancer and chromosomal location of amplified DNA sequences

Case	Age	Sex	Pathological stage T	N	M	Stage	Amplification
L1	67	M	4	2	0	IIIb	3q26.1–q26.3
L6	69	M	2	0	0	I	–
L10	65	M	2	0	0	I	1q,3q26.1–q26.3,5p,6p21.1
L12	68	M	3–4	x	0	IIIb	–
L13	65	M	2	0	0	I	–
L22	75	M	4	2	0	IIIb	–
L26	65	M	4	2	0	IIIb	3q26.1–q26.3
L27	54	M	3–4	2	0	IIIb	–
L38	73	M	3–4	x	x	nd	–

T, tumour; M, metastasis; N, node.

long arm, is consistent with the idea of isochromosome 3q in a subset of SCLC.

Although alterations of chromosome 3 appear to be a hallmark of lung tumours, the underlying molecular mechanisms are likely to differ in SCLC and NSCLC. In contrast to SCLC, allelic loss is frequently found not only for the short arm of chromosome 3, but also for the long arm [27]. In addition, alleotyping indicates differences in the tumorigenic mechanisms of SCC and adenocarcinoma as the major histological subtypes of NSCLC [27, 28]. Furthermore, our study failed to reveal an over-representation of the entire long arm in any of the analysed SCCs, but indicated a specific amplification site at 3q26.1–q26.3. In SCLC, there is only one case reported to carry a specific amplification on 3q [19]. However, the regional assignment of this amplified domain that falls within 3q27–28 differs from the localisation of the amplification identified in SCC (3q26.1–q26.3). Taken together, these data indicate that a specific amplified gene within 3q26 is likely to play a causative role in the pathology of SCC, but not necessarily in adenocarcinoma and SCLC.

The high incidence of DMs in 77% of NSCLC provides further evidence for DNA amplification as an important mechanism in tumour development [7]. As amplifications of single genes are not necessarily demonstrated by reverse chromosome painting, the percentage of NSCLC carrying an amplified gene at 3q26.1–q26.3 might be higher than indicated in our study (3/9 cases). As shown by Joos and colleagues [17], the detection depends not only on the amplification level, but also on the extension of the amplified domain.

Importantly, the amplification at 3q26.1–q26.3 was found in all SCCs that were identified as carrying an amplified domain by reverse chromosome painting. This finding strongly indicates a key role for this chromosomal region in the development of SCC.

Although there are few reports on specific amplified domains in NSCLC, e.g. *PTHRT* and *KRAS*, none of these genes reside within region 3q26.1–q26.3. To our knowledge, no oncogenes have been mapped on this region. Further studies are warranted to delineate which specific genes are amplified in SCC at 3q26.1–q26.3.

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