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A comprehensive search for DNA amplification in lung cancer identifies inhibitors of apoptosis cIAP1 and cIAP2 as candidate oncogenes

Zunyan Dai[1,2], Wei-Guo Zhu[3], Carl D. Morrison[1,2], Romulo M. Brena[1,6], Dominic J. Smiraglia[1], Aparna Raval[1], Yue-Zhong Wu[1], Laura J. Rush[1,4], Patrick Ross[5], Julian R. Molina[7], Gregory A. Otterson[3] and Christoph Plass[1,*]

[1]Program of Human Cancer Genetics, Department of Molecular Virology, Immunology and Medical Genetics, [2]Department of Pathology, [3]Division of Hematology/Oncology, Department of Internal Medicine, [4]Department of Veterinary Biosciences, [5]Department of Clinical Surgery, [6]Department of Molecular Genetics and the Comprehensive Cancer Center, The Ohio State University, Columbus, OH, 43210, USA and [7]Department of Oncology, Mayo Clinic, Rochester, MN 55905, USA

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Amplification of oncogenes is an important mechanism that can cause gene overexpression and contributes to tumor development. The identification of amplified regions might have both prognostic and therapeutic significance. We used primary lung carcinomas and lung cancer cell lines for restriction landmark genomic scanning (RLGS) to identify novel amplified sequences. Enhanced RLGS fragments that indicate gene amplification were observed in primary tumors and lung cancer cell lines of both non-small cell lung cancer and small cell lung cancer. We identified one novel amplicon on chromosome 11q22, in addition to previously reported amplicons that include oncogenes MYCC, MYCL1 and previously identified amplification of chromosomal regions 6q21 and 3q26–27. Amplification of 11q22 has been reported in other types of cancer and was refined to an ~1.19 Mbp region for which the complete sequence is available. Based on a patient sample with a small region of low-level amplification we were able to further narrow this region to 0.92 Mbp. Genes localized in this region include two inhibitors of apoptosis (cIAP1 and cIAP2). Immunohistochemistry and western blot analysis identified cIAP1 and cIAP2 as potential oncogenes in this region as both are overexpressed in multiple lung cancers with or without higher copy numbers.

INTRODUCTION

Gene amplification, as a mechanism leading to overexpression of proto-oncogenes, plays a major role in cancer development. Numerous oncogenes have been identified and their characterization has led to a better understanding of tumorigenesis (1). Activated oncogenes are also important prognostic factors (2). Examples include HER2/neu in breast cancer (3), MYCN in neuroblastoma (2) and MYCC in small cell lung cancer (4). In addition, amplified oncogenes are potential molecular targets for cancer treatment, as recently demonstrated for HER2/neu in breast cancer (5).

Amplification of the MYC-family of proto-oncogenes (MYCC, MYCN and MYCL1) in human lung cancers has been well characterized (6). MYCC is amplified in both small cell lung cancers (SCLC) and non-small cell lung cancers (NSCLC), while MYCN and MYCL1 amplifications are usually limited to SCLC (6). Although MYC gene amplification is a rare event in primary lung tumors, it is more frequently observed in lung cancer cell lines and primary lung cancer following chemotherapy (4,7). Other oncogenes in lung cancer are also amplified at low frequency (8) and include cyclin D1, EGFR, HER2/neu and MDM2 (9–11). However, the decoy receptor 3 (DcR3) gene, located on chromosome 20q13 that blocks FAS ligand induced apoptosis, was found to be amplified in eight out of 16 primary lung tumors (12).

The chromosome 3q26–27 amplicon has recently been identified in non-small cell lung carcinoma by comparative

*To whom correspondence should be addressed at: The Ohio State University, Division of Human Cancer Genetics, Medical Research Facility 464A, 420 West 12th Ave., Columbus, OH 43210, USA. Tel: +1 6142926505; Fax: +1 6146884761; Email: plass-1@medctr.osu.edu

genomic hybridization (CGH) (13,14). This amplicon is observed primarily in squamous cell carcinomas (SCC) of the lung and is rarely found in adenocarcinomas (15,16). Multiple candidate oncogenes including PIK3CA (14) and *amplified in SCC* (AIS), map to this region (17).

Several techniques have been used to identify DNA amplification in cancer, including cytogenetic and/or molecular methods such as comparative genome hybridization (13) and the newly developed CGH on arrayed BAC (14) or cDNA clones (18). Restriction landmark genomic scanning (RLGS), a two-dimensional gel electrophoresis, is a method mainly used for the detection of genome-wide DNA methylation patterns; however, RLGS also has the capacity to perform genome-wide scanning for DNA amplification (19–21). Intensities of RLGS fragments correlate precisely with gene copy numbers since RLGS profiles display labeled restriction ends. The increased copy number leads to an enhanced RLGS fragment as compared to the one found in the normal tissue DNA. RLGS has been successfully used for the identification of DNA amplification in a variety of human primary malignancies (21–25), human cancer cell lines (26–28) and mouse tumors (29,30).

Here we report a genome scan for amplified sequences in lung cancer by RLGS. Four amplified regions have been identified and characterized in primary lung cancers. Among them, three are known amplicons on 1p34, 6q21 and 3q26–27 and the fourth is on 11q22, a region for which no amplification has been reported in primary lung cancers. Detailed characterization of the 11q22 amplicon allowed us to define the amplified region and identify cIAP1 and cIAP2 as candidate oncogenes in lung cancer.

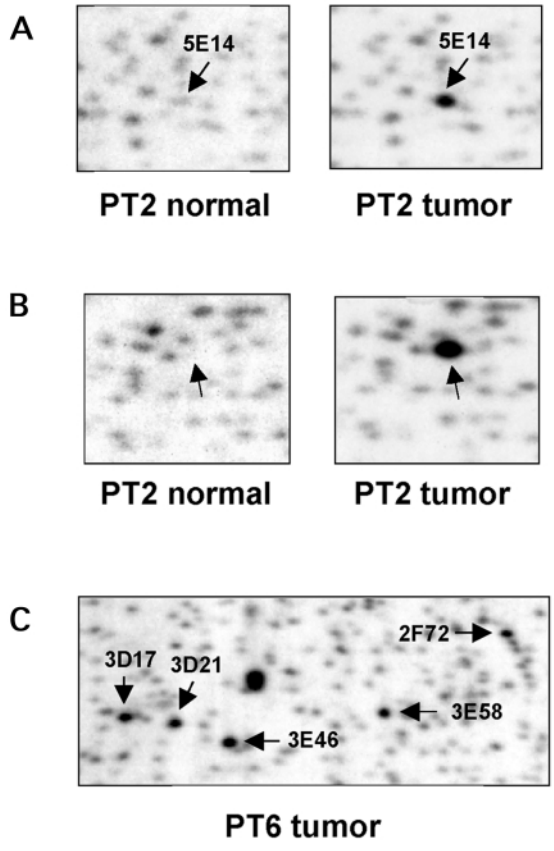

Figure 1. RLGS sections with enhanced fragments in primary lung cancer. (**A**) Amplification of fragment 5E14 (arrow) in PT2. In the RLGS profile from normal adjacent tissue 5E14 has single copy fragment intensity. (**B**) Multi-copy DNA fragment in PT2 tumor profile. This fragment is not present in the normal lung profile. (**C**) A group of RLGS fragments, 2F72, 3D17, 3D21, 3E46 and 3E58, showed increased intensity to the same level in PT6 tumor profile, indicating that they may be derived from the same amplicon.

RESULTS

A genome-wide search for amplified sequences in human lung cancers by RLGS

To identify novel regions of DNA amplification in human lung cancers, 20 paired normal tissue and primary NSCLC, nine NSCLC cell lines, five primary SCLC and eight SCLC cell lines were analyzed by RLGS. RLGS profiles of primary lung cancers were compared with a profile derived from normal lung tissue from the same patient. All enhanced RLGS fragments were named according to RLGS master profile addresses previously established (31). Two types of enhancements were identified in the tumor profiles. The first type was seen in RLGS fragments that were also present in the normal profiles with diploid copy number intensity. For example, the intensity of 5E14 on the normal lung profile of patient (PT) 2 is the same as the intensity of surrounding fragments and represents a single-copy sequence while the intensity of 5E14 is enhanced in the tumor of PT2 (Fig. 1A). The second type of enhancement in tumor profiles was seen in RLGS fragments that were not present in the normal profile (Fig. 1B). The first type represents DNA amplification of a single copy sequence, the second type most likely represents hypomethylation of a normally methylated repeat sequences that contains a *Not*I site or a novel rearrangement (22,32).

The enhanced RLGS fragments of the first type identified in at least one patient are listed in Table 1. Seven out of 20 NSCLC tumors and one of the five SCLC primary tumors showed amplification. RLGS fragments 1G20, 2F51, 3D17, 3D21, 3G68, 5E14 and 5GXX were enhanced in two primary lung cancers, while all other fragments were found only in one tumor. Three NSCLCs (PT1, PT11 and PT14) showed enhancement of only a single RLGS fragment, while the other five tumors (PT2, PT6, PT7, PT12 and PT24) showed multiple enhanced RLGS fragments. RLGS fragments 1G20, 2E46, 2F72, 3E46 3E58, 3D17 and 3D21 were present with low-level enhancement at equal intensity in PT6 (Fig. 1C, showing 2F72, 3E46, 3E58, 3D17 and 3D21), suggesting that they might be derived from a single amplicon. The tumor from PT24 showed enhancement in a subset of these fragments (1G20, 3D17 and 3D21), suggesting that this tumor may have a smaller amplicon size. In addition, the tumor from PT12 showed several amplified sequences (fragments 2F41/42, 2F50, 3D53, 4D13 and 5C25). However, intensities of these fragments varied, suggesting that they may be derived from different amplicons (data not shown).

Each of the lung cancer cell line profiles were compared with two normal lung profiles. Overall, cell lines showed a higher number of enhancements as compared to primary tumors. Among the 17 cell lines, eight out of the nine NSCLC cell lines

Table 1. Enhanced RLGS fragments in primary lung cancers

RLGS fragment	AC PT1	SCC PT2	SCC PT6	LCC PT7	LCC PT11	SCC PT14	SCC PT24	SCLC PT12	NotI-EcoRV clone sequence[a]	Chromosomal location[b]	Related genes, ESTs
3G68	+	+									
5E14		+		+					AP000942.5 (44045–46459)	11q22	LOC143865
2E1		+									
5GXX[c]		+			+						
1G20			+				+		AC117415.6 (92689–96295)	3q26.3–27	SOX-2
2E46			+								
2F72			+						AC068644.12 (155539–158713)	3q26.33–27.1	MRP5
3E46			+								
3E58			+						AC068644.12 (158706–161287)	3q26.33–27.1	MRP5
3D17			+				+		AC078799.21 (51984–53677)	3q26	LOC200816
3D21			+				+		AC007823.37 (143153–144982)	3q26.32	LOC51193
2F51				+		+					
2F41/42								+	AL139260.19 (20339–23614)	1p34.3	LOC163501
2F50								+	AL139158.11 (18113–21073)	1p34.1	POU3F1
3D53								+			
4D13								+	AL359709.15 (18552–20325)	6q21	BM547890, POP3
5C25								+	AL359709.15 (17050–18181)	6q21	BM547890, POP3

AC, adenocarcinoma; SCC, squamous cell carcinoma; LCC, large cell carcinoma; SCLC (PT 12), poorly differentiated neuroendocrine carcinoma of small cell carcinoma and large cell carcinoma.
[a]NCBI database www.ncbi.nlm.nih.gov/.
[b]The chromosomal location was assigned based on the UCSC database http://genome.ucsc.edu/, April 2002 version, but combined information from both NCBI and UCSC databases was used to localize 3D17. 1G20 and 2F50 are based on www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM.
[c]RLGS fragment 5GXX is not present in the RLGS master profile.
SRY-2, related HMG-box gene 2; MRP5, multidrug resistance-associated protein 5; POU3F1, pou domain, class 3, transcription factor 1; POP3, popeye protein 3.

and all eight SCLC cell lines showed amplified sequences (Table 2). The majority of fragments showed low-level (3–5-fold) enhancement on RLGS profiles as measured by phosphorimager quantification. Three cell lines, H23, H125 and H82, showed numerous enhanced sequences at low levels, possibly as a result of whole chromosomal gains, or gains of large chromosomal regions. In contrast to the low-level amplifications seen in the majority of the enhanced RLGS fragments, fragments 3B35 and 4C3 were amplified in cell lines H23, H82, N417 and H792 with copy numbers greater than 20. Seven RLGS fragments (2C59, 2F36, 2G55, 3B35, 3G68, 4C3 and 5C1) were enhanced in more than one cell line (Table 2).

Taken together our analysis identified 17 enhanced RLGS fragments of the type 1 group in primary lung tumors that most likely represent DNA amplification. The RLGS analysis also confirms previous data (7) and identified a higher degree of amplified sequences in lung cancer cell lines, most likely a reflection the overall trend of increased genomic instability in cancer cell lines.

Cloning of RLGS fragments

To further characterize amplified sequences in lung cancer, it was critical to clone and sequence the enhanced RLGS fragments. Subsequent database searches allowed the mapping of sequences to specific chromosomal regions and the identification of genes in the vicinity. Some of the sequences (3B35, 3E24, 3C71, 3G68 and 4C3) had been identified in previous studies (22,28). Fragments 3B35 and 4C3, which are amplified in cell lines H23, H82, N417 (33) and H792, are sequences located in the MYCC gene region on chromosome 8q24 (22). The finding of MYCC amplification in these lung cancer cell lines confirms previous work (33).

In order to identify novel amplified sequences in lung cancer additional fragments of the type 1 group, enhanced in primary lung tumors were cloned. The *Not*I-*Eco*RV plasmid library and corresponding library mixing gels were used to facilitate the cloning (19). Table 1 summarizes the information obtained from database searches of the cloned RLGS fragments. Two groups of sequences could be identified from database and literature searches. The first group includes sequences that fell into known regions of DNA amplification in lung cancers. For example, amplified fragments from tumor PT6 are all located on chromosome 3q26–27, a region known to be amplified in SCC of the lung (14). Fragments 3D17, 3D21 and 1G20 were also amplified in SCC tumor PT24 but 2F72 and 3E58 were not, which indicates that the amplicon in this tumor is smaller than in PT6. Fragments 2F41/42 and 2F50, amplified in PT12, were mapped to chromosome 1p34 ~1 Mbp away from the MYCL1 gene. RLGS fragments 4D13 and 5C25, amplified

Table 2. Enhanced RLGS fragments in lung cancer cell lines

	Diagnosis	Cell line	Enhanced RLGS fragments
NSCLC	AC	H23	2B29, 2D26, 2F9, **2F51**, **3B35**, 3G42, 4B14, **4C3**, 4D29
	AS	H125	1E11, 2E8, 2D31, 2D39, 2D43, 2D44, **2C59**, 3B10, 3D65, 3H6, 3H57, 4C30, 4F7, 5F19, 5G65
	AC	H522	None
	N/A	A549	2D60
	LCC	H1155	**3G68**
	LCC	H1299	2C13, **2C59**, 2F62, 3D9
	AC	H2009	2D23, 3D37
	AC	H2086	2E61, 3E34, 3C71, 3F37, **5C1**, **5E14**
	N/A	H2172	**5C1**, 3C34, 4E10, 4F44
SCLC cell line		H69	**2G55**
		H82	1G35, **3B35**, 3H18, 3H46, **4C3**, 5C40, 5E39, 6C21, 6G13, 6G27
		H209	2D57
		H211	**3G68**
		N417	**2G55**, **3G68**, **3B35**, **4C3**
		H719	**3G68**, **2F36**
		H792	**3G68**, **3B35**, **4C3**
		H841	**3G68**, **2F36**

AC, adenocarcinoma; LCC, large cell carcinoma; AS, adenosquamous. Bold fragments indicate enhancements found in more than one cell line, or shared by primary tumors.

in PT12, are located on chromosome 6q21, a region of amplification in lung cancer, recently identified by CGH (34). The second group of sequences fell into chromosomal regions for which candidate oncogenes had been identified in other types of cancers, but not in lung cancer. For instance, 5E14 was located on chromosome 11q22, a 1.8 Mbp region that has been reported to be amplified in primary esophageal cancers and cancer cell lines as well as other types of cancer (35). To our knowledge, this chromosomal region has not been well characterized in lung cancer.

Confirmation of DNA amplification by Southern hybridization

To confirm that the enhanced RLGS loci represent genomic amplification, probes from either RLGS fragments or adjacent sequences were prepared for Southern hybridizations. A probe for insulin promoter factor 1 (IPF1) gene located on chromosome 13q12.1 was used as DNA loading control for all the hybridizations. We confirmed DNA amplification for four chromosomal regions for which RLGS enhancements had been identified. Southern hybridization for the amplicon on chromosomal region 3q26–27, identified in PT6 and PT24, showed approximately a 5-fold (3D17) or 10-fold (2F72) increased intensity in the tumor of PT6 as compared to the normal adjacent tissue (Fig. 2A). Southern hybridization using probe 2F41/42 for the second amplicon on chromosome 1p34, showed approximately a 7-fold increase in intensity (Fig. 2B). This region includes MYCL1, which was amplified approximately 12-fold. Amplification of chromosome 11q22 was demonstrated using cIAP1 as probe for Southern hybridization (see Fig. 2C, and below). We found that the region was amplified approximately 7-fold in PT2, and 5-fold in cell line H2086. The fourth genomic region on 6q21 showed DNA amplification up to 14-fold in PT12 (Fig. 2D). This region includes the popeye protein 3 (POP3), blood vessel epicardial substance (BVES), and EST LOC135537 and KIAA1320.

Characterization of the 11q22 amplicon

Among the four amplicons described above, the 1p34, 6q21 and 3q26–27 amplicons are known regions of DNA amplification in lung cancer. The amplicon on 11q22 was also found to be amplified in esophageal cancer within a 1.8 Mbp region (35). Although a large amplified region from 11q13 to 11qter has been reported in the NSCLC cell line RVH6489 (36), 11q22 had not been reported previously as a clearly defined amplicon in lung cancer. 5E14 is located in an area on 11q22 that has been completely sequenced (Fig. 3). In order to define the core amplified region, a set of probes (Table 3) were designed for Southern hybridizations (Figs 3 and 4). The intensity of the Southern hybridization signals was quantified using phophorimager and is listed in Figure 4. The amplicon in PT2 was localized to a region extending from LOC143867 to MMP1, spanning 1.19 Mbp. The amplicon in cell line H2086 is at least as large as the one detected in PT2 although its exact size could not be determined (Fig. 4).

PT7 showed low-level amplification of a region localized between markers MMP7 and LOC143868 spanning an interval of 0.92 Mbp and represents the minimal region of overlap. This region includes cIAP1 and cIAP2 as potential candidates oncogenes but not the MMP genes. We performed immunohistochemistry for both genes on nine tumor sections and nine normal sections from the same patients and found overexpression of cIAP1 in five out of nine tumor samples and overexpression of cIAP2 in six out of nine cases (see Fig. 5 for examples and Table 4). In this set of nine tissue sections we included the samples from PT2 and PT7, both characterized by amplification of the region containing cIAP1

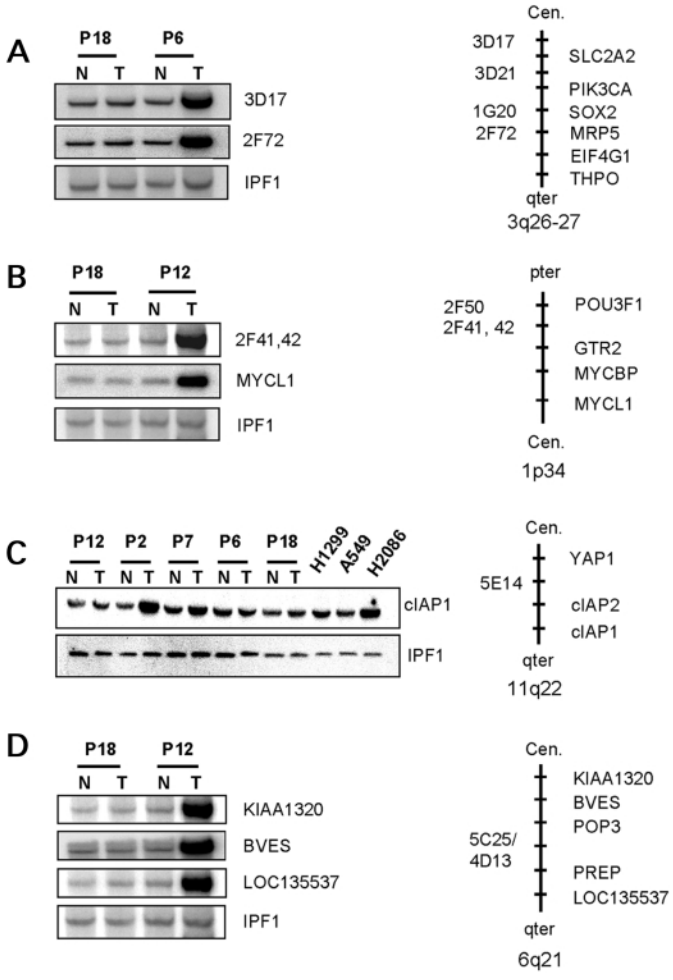

Figure 2. Confirmation of DNA amplification by Southern hybridization. (**A**) Chromosome 3q26–27 amplification was demonstrated using probes from RLGS fragments 3D17 and 2F72. All DNAs were digested with *Eco*RV. The chromosomal map for genes and RLGS fragments was created based on the information from the UCSC database and published literature (60). (**B**) 1p34 amplification shown with probes 2F41/42 and MYCL1 on *Eco*RV digested DNA. (**C**) The 11q22 amplicon was verified using the cIAP1 probe on *Eco*RI digested DNA. (**D**) Confirmation of 6q21 amplification using probe KIAA1320, BVES and LOC135537 on *Eco*RV digested DNA.

and cIAP2. Immunohistochemistry showed that cIAP1 was overexpressed only in PT7 but not in PT2 (Fig. 5B), whereas cIAP2 was found to be overexpressed in both patient samples (Fig. 5A and B). Overall cIAP2 seemed to be expressed at a higher degree (six out of nine samples with >10% cells expressing cIAP1) when compared to cIAP1 with only two samples expressing this gene in >10% of the cells. Importantly, we also identified cIAP1 and cIAP2 overexpression in patients with no evidence of genomic amplification (i.e. PT1, 10, 33 and 35 see Table 4).

We also performed western blot analysis to obtain an independent measure for cIAP1 and cIAP2 overexpression. Seven patient samples (normal and tumor), including PT2 and PT7, were used for western blot analysis. Proteins were hybridized sequentially with cIAP1 and cIAP2 antibodies, with a tubulin antibody as a loading control. The data is shown in Figure 6 and summarized in Table 4. Samples with amplification (PT2 and PT7) as well as without amplification (PT1), showed an increased expression of both cIAP1 and cIAP2. Patients 32 and 34 showed increased expression of cIAP2 but not cIAP1. The analysis of patient 33 protein was inconclusive due to the poor quality of the isolated protein lysate. This data supports the findings by immunohistochemistry that overexpression of both cIAP1 and cIAP2 occurs in cells with and without DNA amplification, suggesting different mechanisms in these cells that contribute to overexpression.

In addition, Southern hybridization was performed on another 44 non-small cell lung cancers to detect cIAP1 gene amplification. Only a single additional tumor with 2.67-fold amplification was detected (data not shown). Together with the RLGS data this indicates that the amplification of 11q22 in lung cancer is infrequent (three out of 64 tumors tested, or 4.5%), similar to the observation in other types of cancer (35).

DISCUSSION

In the present study, we have performed restriction landmark genomic scanning to identify DNA amplification in human lung cancer. The characterization of enhanced RLGS fragments in primary lung cancer identified amplicons on 1p34, 3q26–27, 11q22 and 6q21. A more detailed analysis of the amplicons on 11q22 identified the inhibitors of apoptosis 1 and 2 (cIAP1 and cIAP2) as candidate oncogenes in this region of amplification.

Amplification of 1p34 had previously been described and this amplicon contains the MYCL1 oncogene (33). Amplification of 3q26–27 had been reported in 25–40% of patients with squamous cell carcinoma of the lung (16,37,38), and only very rarely in adenocarcinomas (15,16). Consistent with these published reports, we found amplification of 3q26–27 in two out of eight squamous cell carcinomas. The cloned fragments derived from this amplicon are located in an area extending 30 Mbp, including several candidate oncogenes in lung cancers (14,39,40). For example, PIK3CA, a gene associated with the phosphatidylinositol-3-kinase pathway, is a candidate oncogene in both lung cancer (14) and cervical cancer (41). Amplification of 3q26–27 has been found in a variety of human cancers, including head and neck squamous cell carcinoma (42,43), cervical cancer (41), primary carcinoma of the fallopian tube (44), osteosarcomas (45), ovarian cancer (46,47), and nasopharyngeal carcinoma (48). In head and neck squamous cell carcinomas and in ovarian carcinomas, amplification correlates with tumor progression (42).

Amplification of 6q21, found in SCLC, has also been described in breast cancer (49,50). This amplicon is currently not well defined but includes the known genes prolyl endopeptidase (PREP), blood vessel epicardial substance (BVES) and POP3. PREP is an enzyme involved in the maturation and degradation of peptide hormones and neuropeptides, and displays increased activity in human lung tumors compared to normal lung (51). BVES and POP3 are expressed in heart and skeletal muscle and are essential for coronary vasculogenesis (52). However, currently no candidate oncogene has been characterized.

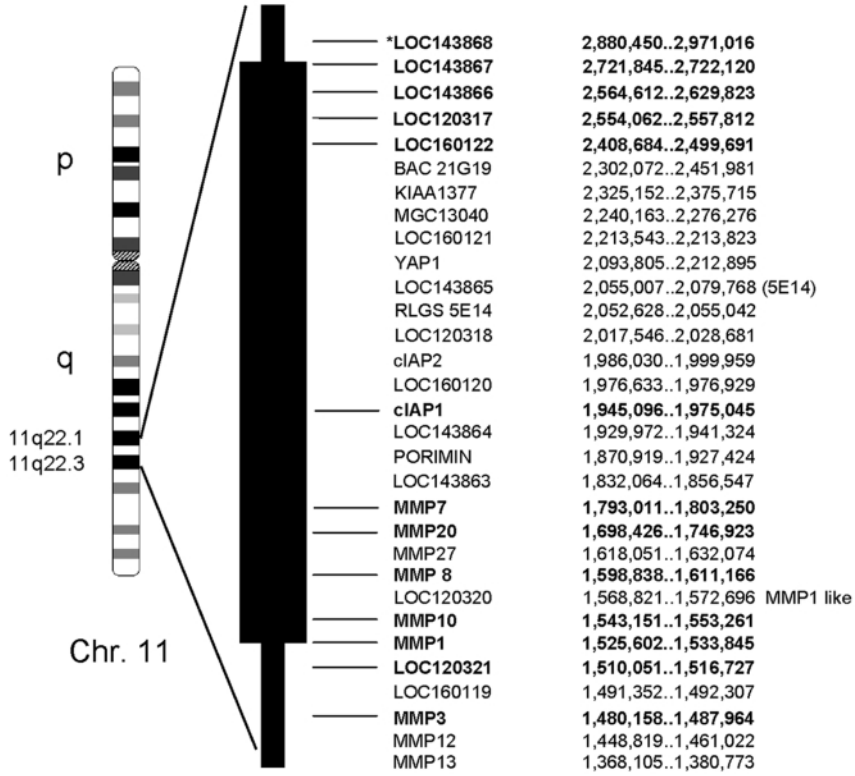

Figure 3. Known genes and ESTs on chromosome 11q22. The black bar indicates the core amplified region in human primary lung cancer. The region has been completely sequenced (contig NT_009151.8). Genes or ESTs marked in bold were used as probes (Table 3) for Southern hybridization. Gene or EST location in the sequence contig is not drawn to scale. Numbers correspond to location of nucleotides within the NT-009151.8 contig.

Table 3. Primers used for probe preparations

Probe name	Forward primer	Reverse primer	Annealing (°C)	Probe size
11q22				
LOC143868	5′-TTGCTTGCCAGTGGTTACAG-3′	5′-CCTGACCAAGCCAATCTGAG-3′	58	497 bp
LOC143867	5′-ACCCCTCTCTATTTCCATTGC-3′	5′-CAGCAGTCTTGTCGGTCTTG-3′	58	410 bp
LOC143866	5′-AGTTGCACCAGGCAGAAGTT-3′	5′-CCTCAGCAACCAGAGGAAAT-3′	58	401 bp
LOC120317	5′-ACAACTTCAGCCAAGGCAGT-3′	5′-CAAGGGCAGGATAAGGAGGT-3′	58	479 bp
LOC160122	5′-TTTCCAGGCTGCTATTTTCC-3′	5′-TGGAGAAGTGCCTCAATGTG-3′	58	505 bp
cIAP2	5′-TTTATCCTAATTTGGTTTCC-3′	5′-AATTCTTAAAGGTTAACTC-3′	50	253 bp
cIAP1	5′-ACATTCATGTTCTAGTCTGC-3′	5′-TTTATTGAGATGTTTCTCAC-3′	50	366 bp
MMP7	5′-TCTTCCAGAGGGTGTCTTGG-3′	5′-AGGATAGGAATCCAGCAGCA-3′	58	603 bp
MMP20	5′-CTTGCTCATGCTCAGTCTCG-3′	5′-CCTTTGCCTGAGCTCGTAAC-3′	58	602 bp
MMP8	5′-GAGCAGAAATGGAAGCGTCT-3′	5′-ACCACATCCTCCAGAAGCAG-3′	58	600 bp
MMP10	5′-TTATTCCTCGTTGCTGCTCA-3′	5′-TCTCCCCTCAGAGTGCTGAT-3′	58	468 bp
MMP1	5′-ATGCTGAAACCCTGAAGGTG-3′	5′-AAGAGGCCAACAGACTTCCA-3′	58	501 bp
LOC120321	5′-AGGCCAAGTGTGAAGAGGAA-3′	5′-TTTGCCCCTTTATGAAGCAC-3′	58	398 bp
MMP3	5′-ACCATTTGGGCTTCTCCTCT-3′	5′-GAGTGTCGGAGTCCAGCTTC-3′	58	506 bp
3q26–27				
3D17	5′-GGAGAGAAAACACGCACACA-3′	5′-CTGCTTCTCCAGTCCTGAGC-3′	58	455 bp
1p34				
MYCL1	5′-GGCATGCAGAAAGGCTTATT-3′	5′-TGCAATCACCCAGAGTGGTA-3′	58	542 bp
6q21				
LOC135537	5′-CGGCTTCAAGTATTGGCATT-3′	5′-GAGGCCTCATCAGTTTCAGC-3′	58	504 bp
BVES	5′-CCTCACTCCTCCCTTCCCTA-3′	5′-ACCAACCCAACTGCAAAACA-3′	58	502 bp
KIAA1320	5′-GCTTACCTGCCCGTAACAAA-3′	5′-GCATGCCAGAAATTGATGTG-3′	58	441 bp

To our knowledge amplification of 11q22 had only been described in a NSCLC cell line, RVH6489, extending from 11q13 to 11qter (36). This indicates that DNA amplification of this sequence is a rare event. We have found amplified 11q22 sequences in two of 20 tumors by RLGS, however when we tested 44 additional samples by Southern blot analysis we did find amplification in one primary lung cancer, supporting the notion that DNA amplification of 11q22 is a rare event in lung

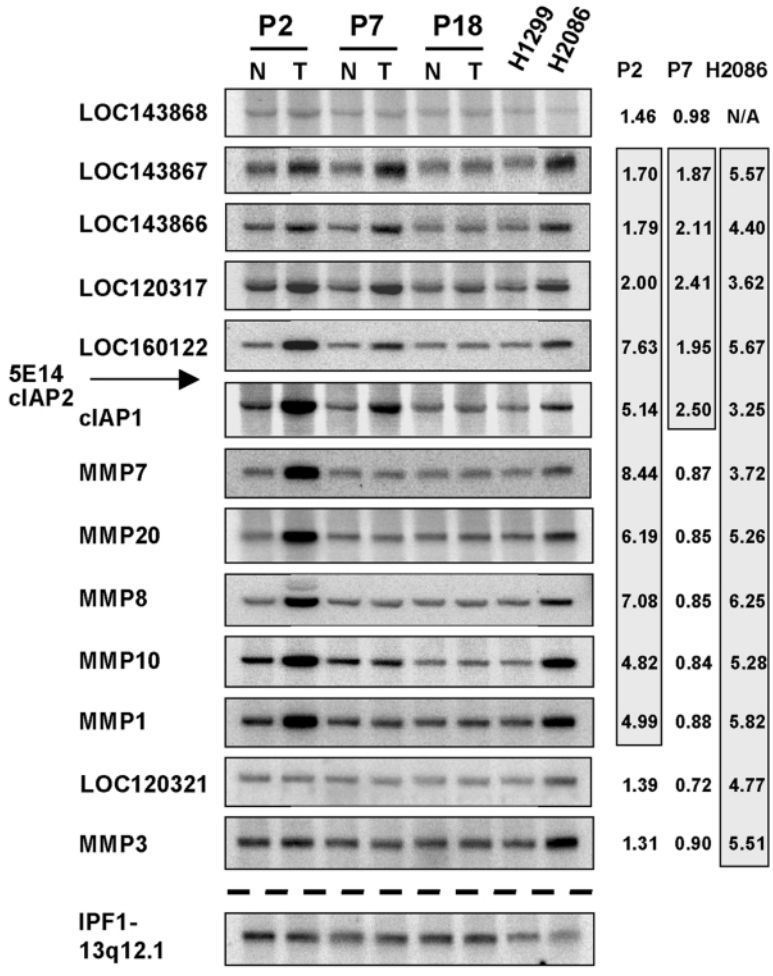

Figure 4. 11q22 amplicon definition by Southern hybridization. All hybridizations were carried out on the same Southern blot with *Eco*RI digestions using probes described in Table 3. The degree of amplification was calculated by densitometry as described in the Materials and Methods. The shaded area indicates sequences that were enhanced 1.5-fold or higher.

cancer and thus has been missed in previous studies. The amplicon was previously described in esophageal squamous cell carcinoma cell lines KYSE2270 and KYSE170 and primary tumors (35). The authors described amplification in BACs RP11-21G19 to 23P6. Based on the current human sequences in this region (NT_009151.8), this 1.1 Mbp region is approximately located between LOC160122 and MMP13 (see also Fig. 3). Therefore, the region that we identified in PT2 is overlapping with the amplicon in esophageal squamous cell carcinoma.

Several lines of evidence support the hypothesis that cIAP1 and cIAP2 are strong candidate oncogenes in this region. First, both genes are localized in the core amplified region defined by two patient samples and one lung cancer cell line. Furthermore, both genes are located in the core-amplified region in esophageal cancers. Second, cIAP1 and cIAP2 are genes that promote cell survival after apoptoic stimuli and thus support tumor growth. The way they achieve the antiapoptotic activity is by the inhibition of specific caspases (53). Both cIAP1 and cIAP2 prevent the proteolytic processing of pro-caspases -3, -6 and -7 by inhibiting the cytochrome c induced activation of pro-caspase-9 (54). Third, we showed by immunohistochemistry and western blot analysis that both genes are overexpressed at high frequencies in lung cancers. Overexpression is not only seen in tumor samples that do have additional copies of the genes but also in tumors that show no DNA amplification. This indicates that these genes are overexpressed in tumor cells by independent mechanisms. It is currently unclear what these mechanisms are, but they probably function either at the transcriptional or posttranslational level. Fourth, it is interesting to note that recently the inactivation of the apoptosis protease activating factor-1 (APAF1) was described in metastatic melanomas (55). APAF1 binds to cytochrome c and this complex promotes the activation of caspase-9. Inactivation of APAF1 thus represents an alternative way of preventing cell death and shows the importance of this pathway in normal cell development. It needs to be determined if this alternative way of disrupting the apoptotic pathway is used in lung cancers. Fifth, based on expression studies as well as functional analysis, cIAP1 has been proposed as candidate oncogene in the 11q22 amplicon in esophageal squamous cell carcinoma (35). Ectopic expression of cIAP1 in mammalian

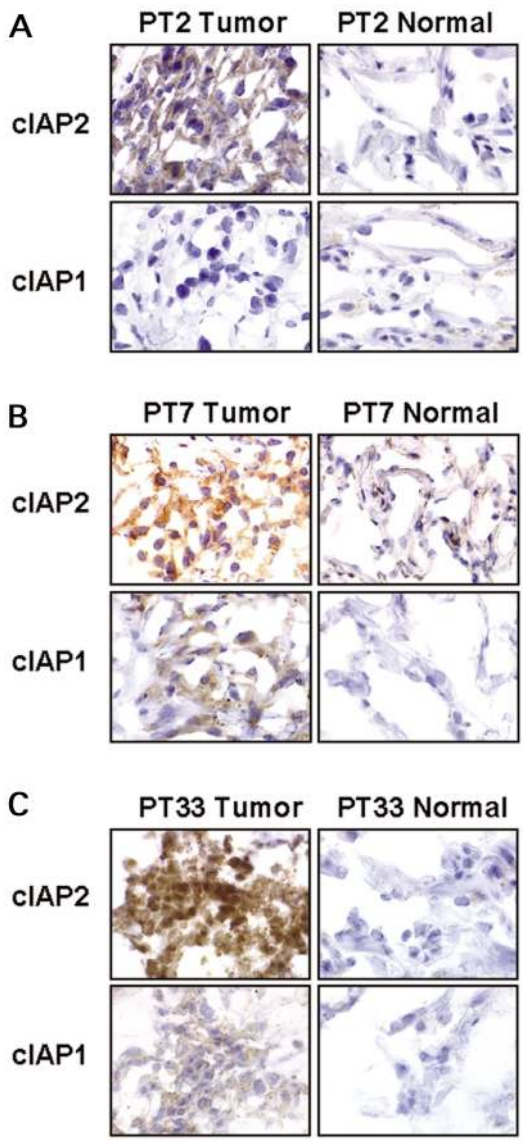

Figure 5. Immunohistochemical staining for both cIAP1 and cIAP2 using frozen sections from PT2 (**A**), PT7 (**B**) and PT33 (**C**) of non-small cell carcinoma of the lung and matching normal tissue. Top lane tumor (left) and normal (right) stained with cIAP2. Bottom row tumor (left) and normal (right) stained with cIAP1 (magnification, ×600). (A) PT2: moderate immunoreactivity for cIAP2 (2+) with a cytoplasmic pattern of staining in a large cell carcinoma and absent staining for cIAP2 in the matching normal lung. Negative immunoreactivity for cIAP1 in the same case with absent staining for cIAP1 in the matching normal lung. (B) PT7: strong immunoreactivity for cIAP2 (3+) with a cytoplasmic pattern of staining in a squamous cell carcinoma and weak to absent (0/1+) staining for cIAP2 in the matching normal lung. Moderate immunoreactivity for cIAP1 (2+) with a cytoplasmic pattern of staining in the same case absent staining for cIAP1 in the matching normal lung. (C) PT33: strong immunoreactivity for cIAP2 (3+) with a cytoplasmic and nuclear pattern of staining in the neoplastic cells and (B) absent staining for cIAP2 in the matching normal lung. Weak immunoreactivity for cIAP1 (1+) with a cytoplasmic pattern of staining in the same case with absent staining for cIAP1 in the matching normal lung.

cells inhibited apoptosis induced by serum deprivation (56). It was also shown that expression of cIAP1 resulted in the resistance of apoptosis induced by chemotherapeutic agents (35), and correlated with radiation resistance in cervical cancer

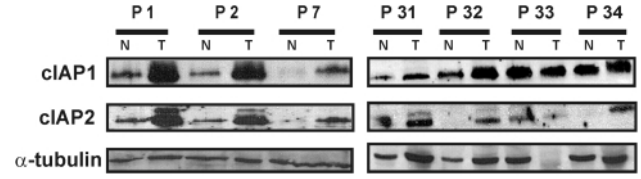

Figure 6. Detection of cAIP1 and cAIP2 by western analysis. Protein detection was performed on human lung tissue protein lysates. Equal amounts of protein were loaded onto each lane. Increased expression of cAIP1 is observed in the tumor samples from patients 1, 2 and 7, with respect to their normal adjacent tissue counterparts. Increased expression of cAIP2 in tumor samples compared to their normal adjacent tissue counterparts is observed in patients 1, 2, 7, 32 and 34. No analysis could be performed on patient 33 due to the poor quality of the isolated protein lysate.

Table 4. Summary of immunohistochemistry and western blot results for cIAP1 and cIAP2

Patient no.	Sample	cIAP1	cIAP2	Western blot[a]		DNA amplification
				cIAP1	cIAP2	
1	Normal	0	0			–
	Tumor	**3**	**3**	Increased	Increased	–
2	Normal	0	0			–
	Tumor	0	**2**	Increased	Increased	++
7	Normal	0	0			–
	Tumor	**3**	**3**	Increased	Increased	+
10	Normal	1	1			–
	Tumor	1	**2**	NA	NA	–
31	Normal	0	1			–
	Tumor	0	1	Normal	Normal	–
32	Normal	0	1			–
	Tumor	0	1	Normal	Increased	–
33	Normal	0	0			–
	Tumor	1	**3**	NA	NA	–
34	Normal	0	0			–
	Tumor	1	1	Normal	Increased	–
35	Normal	0	0			–
	Tumor	1	**2**	NA	NA	–

Bold indicates samples with high expression in over 10% of the cells as graded 2 and 3.
PT2 and PT7 showed DNA amplification, at low (+) or high degree (++).
[a]Relative abundance of cIAP1 and cIAP2 proteins per tumor sample compared to its normal counterpart. NA, not analyzed. Normal: not increased expression compared to normal tissue.

(57). In addition, cIAP2 was overexpressed in human malignant pleural mesothelioma and the high expression of cIAP2 correlated with drug resistance in cancer cells (58).

In conclusion, and based on our current understanding of cIAP1 and cIAP2, as well as our finding of overexpression, both cIAP genes are strong candidate oncogenes in lung cancer.

MATERIALS AND METHODS

Primary lung cancer tissue samples and cell lines

Primary human lung cancer and normal adjacent tissue samples were collected through the Cooperative Human Tissue Network at The Ohio State University, James Cancer Hospital. A total of 20 NSCLCs were studied, 16 of them had previously been used

for DNA methylation profiling (59). These tumors included eight squamous cell carcinomas, six adenocarcinomas, four large cell carcinomas and two uncharacterized NSCLC. In addition, five SCLCs were obtained from the Mayo Clinic (Rochester, MN, USA). Tissue specimens were procured with IRB approval.

Nine non-small cell lung cancer cell lines, H23, H125, H522, H1155, H1299, H2009, H2086, H2172 and A549, and eight SCLC cell lines, H69, H82, H209, H211 H719, H792, H841 and N417, were obtained from ATCC and cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 100 units/ml Penicillin and 0.1 mg/ml Streptomycin (Invitrogen).

Restriction landmark genomic scanning

DNA isolation and RLGS was performed according to published protocols (19,59). In summary, randomly fragmented ends of high molecular weight DNA were blocked using DNA polymerase I (Boehringer Mannheim), followed by a restriction digestion of the DNA with *Not*I (Promega, Madison, WI, USA). *Not*I restriction ends were labeled using [α-^{32}P]dGTP and [α-^{32}P]dCTP (Perkin Elmer, Foster, CA, USA) in the presence of Sequenase (Amersham, Piscataway, NJ, USA). The DNA was further digested with the restriction enzyme *Eco*RV (Promega). *Not*I-*Eco*RV DNA fragments were separated by molecular weight through an 0.8% agarose gel (first dimension). After an in-gel digestion using the restriction enzyme *Hinf*I (New England Biolabs, Beverly, MA, USA), the second dimension separation was carried out on a 5% polyacryamide gel. The dried gels were exposed to X-ray film for 5–10 days. The RLGS profiles of primary lung tumors and normal adjacent tissue from the same patients were superimposed to detect enhanced RLGS fragments that might represent amplified DNA. The RLGS profiles were exposed to a phosphorimager screen for quantification. All the RLGS fragments were named according to the master profile (see online: http://pandora.med.ohio-state.edu/masterRLGS). Fragments not present in the master profile were named according to the section in which they were found (e.g. 5GXX).

RLGS fragment cloning and sequencing

Clones corresponding to RLGS fragments were identified using the *Not*I–*Eco*RV plasmid library and library RLGS mixing gels as previously described (19). Confirmed plasmid clones were sequenced in the Core Facility of the Division of Human Cancer Genetics using M13 forward primer. The NCBI database, www.ncbi.nlm.nih.gov/, and UCSC human genome project working draft database, http://genome.ucsc.edu/, were searched for sequence homologies and chromosomal locations.

Southern hybridization

Probes were made from either restriction enzyme digested fragments of the plasmid clones or from PCR products. The IPF1 probe was a 493 bp *Xba*I–*Eco*RV fragment; the 5E14 probe was a *Xba*I–*Eco*RV fragment of 1426 bp; the 2F72 was a 381 bp *Pst*I–*Bam*H1 fragment; and the 2F41/42 probe was a 278 bp *Bgl*II–*Not*I fragment. All other probes were made by PCR amplification of genomic DNA with primers and conditions listed in Table 3. One microliter of the PCR product was used as template for a second round of PCR amplification. PCR product was purified in an agarose gel and eluted using QIAquick® gel extraction kit (Qiagen, Valencia, CA, USA). All the probes were labeled with [α-^{32}P]dCTP using Prime-It® II Random Primer labeling kit (Stratagene). Southern hybridization followed the published protocol (19). The Southern blots were exposed to phosphorimager screen and quantified.

Immunohistochemical staining

Immunoperoxidase staining was performed on frozen tissue cut at 4 µm intervals and placed on positively charged slides. Slides with specimens were fixed in acetone for 6 min and stored frozen until ready for use. Slides were then rewarmed to room temperature and rehydrated with PBS for 6 min. All slides were quenched for 5 min in a 3% hydrogen peroxide solution in water to block for endogenous peroxidase prior to immunostaining and then rinsed in tap water. No antigen retrieval was performed. The primary antibodies used were cIAP1 (clone H-83, Santa Cruz, CA, USA), no.SC-7943, 1:50) and cIAP2 (clone H-85, Santa Cruz, CA, USA), no.SC-7944, 1:50). Slides were then placed on a Dako Autostainer and incubated for 60 min at room temperature with the specific primary antibodies for cIAP1 or cIAP2. The detection system used was a labeled streptavidin–biotin complex. This method is based on the consecutive application of (1) a primary antibody against the antigen to be localized, (2) biotinylated linking antibody, (3) enzyme conjugated streptavidin, and (4) substrate chromogen (DAB). Slides were then counterstained in Richard Allen hematoxylin, dehydrated through graded ethanol solutions and coverslipped. Negative controls for both cIAP1 and cIAP2 were done by the same procedure as listed above with the omission of the primary antibody.

The slides were examined by a pathologist (C.M.) and reviewed with the primary investigators (Z.D., C.P.) with immunoreactivity defined as more than 1% of cells staining. For both cIAP1 and cIAP2 positive results were interpreted as a granular cytoplasmic or nuclear pattern of immunoreactivity. The immunohistochemical staining was graded and recorded as 1+ (1–10% positive cells), 2+ (10–50% positive cells), or 3+ (>50% positive cells). Negative controls were stained appropriately.

Western blot analysis

Human lung samples were crushed in lysis buffer [50 mM Tris–HCl, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.1% Igepal CA-630, with protease and phosphatase inhibitor cocktails (Sigma)]. Equal amounts of protein (50 µg) were separated on 7.5% SDS–PAGE and transferred onto Hybond™ ECL™ nitrocellulose membranes (Amersham Pharmacia Biotech, Uppsala, Sweden). The membranes were incubated in blocking buffer [5% non-fat milk, 200 mM NaCl, 25 mM Tris (pH 7.5), and 0.05% Tween 20] for at least 2 h and then with primary rabbit polyclonal antibodies against cAIP1 or cAIP2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) 1:200 at 4°C overnight with shaking or with primary mouse monoclonal

antibody against α-tubulin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) 1:2000 at 4°C for 2 h. After washing with TBS-T (20 mM Tris, 500 mM NaCl and 0.1% Tween 20) once, blocking buffer once and then TBS-T four times, 5 min each, membranes were incubated with the appropriate secondary antibody (antirabbit 1:2000 for cAIP1 and cAIP2 antibodies and antimouse 1:2000 for α-tubulin antibody) at 4°C for 1–2 h. After the same wash for primary antibody, proteins were detected using a chemiluminescent detection system (Amersham Pharmacia Biotech).

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