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SHORT COMMUNICATION

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GPC5 is a possible target for the 13q31-q32 amplification detected in lymphoma cell lines

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Abstract Comparative genomic hybridization (CGH) analyses have detected gains of copy number on 13q, especially at 13q31-q32, in cell lines and primary cases of various types of lymphoma. Since amplification of chromosomal DNA is one of the mechanisms that can activate tumor-associated genes, and because 13q amplification had been reported in various other types of tumors as well, we attempted to define by fluorescence in situ hybridization (FISH) a common region at 13q31-q32 in which to explore genes that might be targets for the amplification events. Although the commonly amplified region we defined was relatively large (approximately 4 Mb), only one true gene, *GPC5*, was found there. *GPC5* was over-expressed in lymphoma cell lines that had shown amplification, in comparison with those that had not. Our findings suggest that *GPC5* is a likely target for amplification, and that over-expression of this gene may contribute to development and/or progression of lymphomas and other tumors.

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Introduction

Gene amplification is frequently observed in human tumors, and appears to be one of the mechanisms leading to activation of proto-oncogenes (Stark et al. 1989). Defining and characterizing genes within amplified chromosomal regions represents an excellent route toward identification of elements involved in tumorigenesis.

Lymphoma remains incurable even with current therapeutic modalities. Multiple genetic alterations that have been identified in various types of lymphoma (Neat et al. 2001; Allen et al. 2002; Mao et al. 2002; Mehra et al. 2002) may be linked in a sequence of events that parallels malignant progression. The *REL* and *BCL2* proto-oncogenes are known amplification targets that are associated with malignant progression of lymphomas (Houldsworth et al. 1996; Rao et al. 1998). However, those two genes do not fully account for all of the genetic material present in amplified regions or for the variety of malignant phenotypes that lymphomas can exhibit. Indeed, cumulative results of comparative genomic hybridization (CGH) analyses have revealed additional amplified regions in lymphomas that contain novel target genes that could be associated with pathogenesis of this disease. (Neat et al. 2001; Allen et al. 2002; Mao et al. 2002). Identification of those target genes should yield new insights into the molecular pathogenesis of lymphoma, establish diagnostic markers, and suggest novel therapeutic targets.

Among the chromosomal regions showing recurrent amplification in CGH analyses of various types of lymphoma, 13q31-q32 is of particular interest because (1) gains of copy number are frequent in that region; e.g., in eight (18%) of 45 cases of follicular lymphoma (Neat et al. 2001) and four (29%) of 14 cases of primary

cutaneous B-cell lymphoma (Mao et al. 2002); and (2) genomic alterations within or around this region have also been implicated in other malignancies such as small and non-small cell lung cancers, neuroglial tumors, and squamous cell carcinoma of the head and neck (Knuutila et al. 1998). Therefore, the 13q31-q32 region may harbor one or more genes which, when activated by amplification, might be associated with progression and/or specific phenotypes of lymphomas and other tumors. However, no actual or potential targets of the amplification at 13q31-q32 have yet been clarified. These observations prompted us to perform a detailed molecular characterization of the 13q31-q32 amplicon using lymphoma cell lines, with the goal of identifying gene(s) involved in the pathogenesis of this type of tumor.

Fig. 1a Representative images of FISH analysis on metaphase chromosome from KARPAS1718, OCI-LY3 and AL-1 with BAC clone 487A02, containing the *GPC5* gene, as a probe. *KARPAS1718* shows remarkable increase in signals with HSR pattern and *OCI-LY3* shows four signals, whereas *AL-1* shows two signals (no amplification). **b** The amplicon map of the 13q31-q32 region between two makers, RH53181 and D13S197. The position of each STS and each of the 17 BACs was compiled from information archived by the UCSC and the NCBI (*left*). This region contains the only known gene, *GPC5* (*middle*). Summarized results of DNA sequence copy-number analysis by FISH in four lymphoma cell lines showing amplification of this region (*right*). The *horizontal axis* shows the number of FISH signals achieved with the BAC probes indicated *above*. The number of signals was truncated at 15 because it was difficult to enumerate them above this level. The smallest region of overlap (SRO) with maximal amplification is indicated by the *closed arrow*. **c** Relative expression of *GPC5* in six lymphoma cell lines, four with increased copy number of *GPC5* and two with normal copy number of *GPC5*. The highest expression is found in the KARPAS1718 cell line exhibiting remarkable copy number gain of this gene (**a**, **b**)

Materials and methods

Cell lines

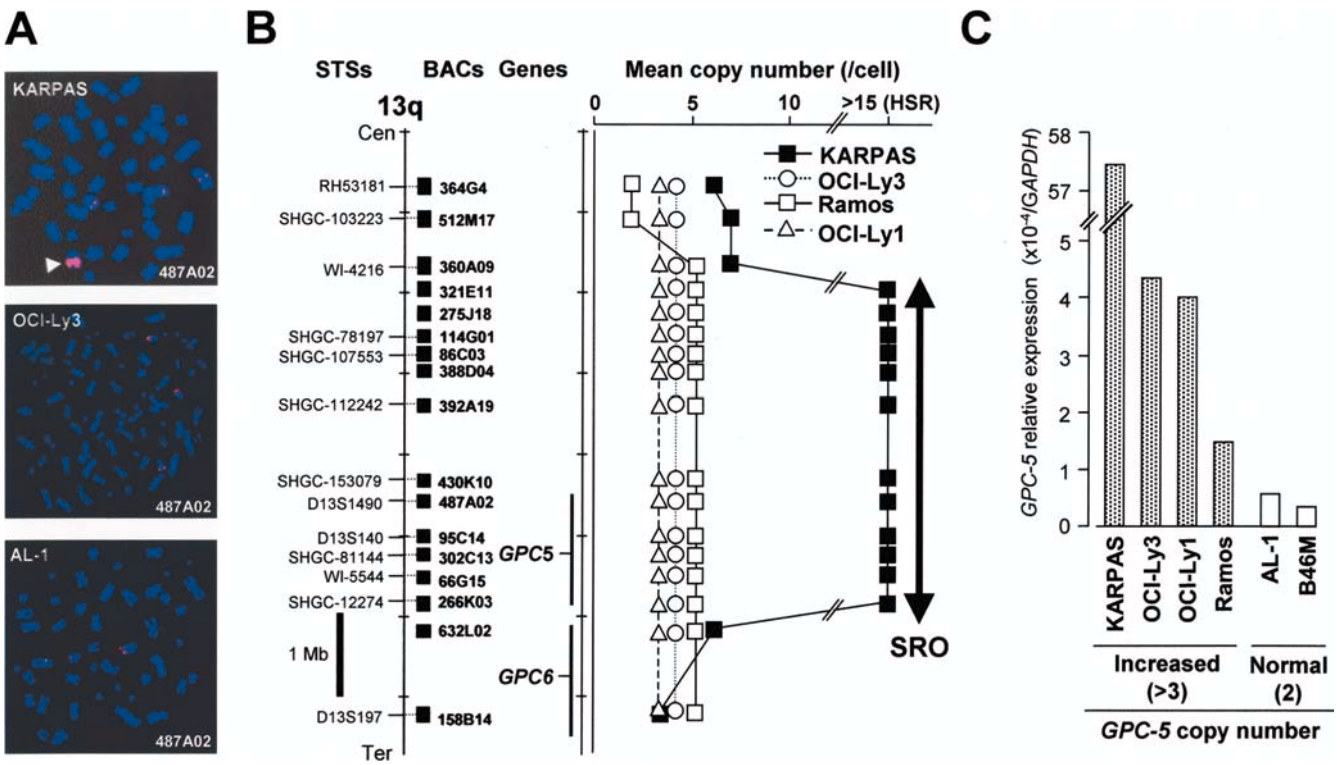
A total of six lymphoma cell lines, KARPAS1718 (splenic lymphoma with circulating villous lymphocyte), RAMOS (Burkitt lymphoma), AL-1 (Burkitt lymphoma), B46M (Burkitt lymphoma), OCI-ly1 (non-Hodgkin lymphoma), and OCI-ly3 (non-Hodgkin lymphoma) were employed in the present study. These cell lines were maintained in RPMI-1640 supplemented with 10% fetal calf serum and penicillin-streptomycin. Metaphase chromosome slides were prepared and used in FISH experiments in the manner described previously (Fukuda et al. 2000).

Fluorescence in situ hybridization (FISH)

We performed FISH analysis using 25 bacterial artificial chromosomes (BACs, 17 are listed in Fig. 1b), as described previously (Inazawa et al. 1993; Ariyama et al. 1999). The locations of BACs within the region of interest were compiled from information archived by the University of California at Santa Cruz (UCSC; http://genome.ucsc.edu/) or the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/). Relative positions of the selected BACs on a map of the 13q31-q32 region are indicated in Fig. 1b. Probes were labeled by nick-translation with biotin-16-dUTP or digoxigenin (Roche Diagnostics, Tokyo, Japan) and hybridized to metaphase chromosomes. Precise localization of each BAC was confirmed using normal metaphase chromosomes.

Reverse transcriptase-polymerase chain reaction (RT-PCR), direct sequence, Northern blot, and database search

We performed reverse transcriptase-polymerase chain reaction (RT-PCR) using at least two primer sets for each of 15 uncharacterized, predicated transcripts within the smallest region of overlap



within 13q31-q32 that we defined. Primer sequences are available on request. Single-stranded cDNAs were generated from total RNAs of cell lines using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, Calif.) according to the manufacturer's directions. RT-PCR was performed using FastStart Taq DNA polymerase (Roche Diagnostics, Tokyo, Japan) according to the manufacturer's directions. Every RT-PCR product was evaluated by electrophoresis, direct sequence using ABI377 Autosequencer, and/or Northern blotting (Human 12-lane MTN, Clontech, Palo Alto, Calif.) described elsewhere (Imoto et al. 2001). All 15 uncharacterized, predicated transcripts were evaluated using NCBI database, such as LocusLink with Evidence Viewer (http://www.ncbi.nlm.nih.gov/LocusLink) and BLAST (http://www.ncbi.nlm.nih.gov/BLAST/).

Real-time quantitative RT-PCR

The quantification of *GPC5* mRNA was carried out using a real-time fluorescence detection method (Wittwer et al.1997). Single-stranded cDNAs were generated from total RNAs of each cell line. Real-time quantitative PCR was performed using LightCycler (Roche Diagnostics) with CYBR Green according to the manufacturer's protocol. The primer sequences for *GPC5* gene were as followings: the forward primer, 5′-GGTCGTTGGAAGAA CTCTCG-3′ and the reverse primer, 5′-GCGGCCACAAATCC TATTTA-3′. The *glyceraldehyde-3-phosphate dehydrogenase* gene (*GAPDH*; Roche Diagnostics) served as an endogenous control and each sample was normalized on the basis of its *GAPDH* content.

Results and discussion

Definition of the 13q31-32 amplicon by FISH

As the first step toward exploring candidate gene(s) we constructed a map of the 13q31-q32 amplicon by FISH, using available lymphoma cell lines, to define a relatively small chromosomal region that would make a positional search possible. The criterion we used to define the amplicon was based on the premise that the best candidates (i.e., targets that are likely to be selected during the amplification process) would be located within the smallest region of overlap among regions showing the highest copy numbers in a panel of cell lines or primary tumors (Albertson et al. 2000).

We first performed FISH analyses using ten BACs located around 13q31-q32, covering approximately 10 Mb, in the six lymphoma cell lines; four of those lines (KARPAS1718, RAMOS, OCI-ly1, and OCI-ly3) had shown copy-number gains of chromosome 13q in G-banding experiments (Mehra et al. 2002) or in CGH studies (Mehra et al. 2002; Dr. Masao Seto in Aichi Cancer Center Research Institute, personal communication) and we found that two BACs, 86C3 and 302C13, showed a consistent increase in the number of signals in these four cell lines (data not shown).

Then we performed FISH analyses using additional 15 BACs located around 86C3 and 302C13 (Fig. 1b). In KARPAS1718 cells, 12 of the 17 BACs used in the second round of FISH experiments (321E11, 275J18, 114G01, 86C3, 388D04, 392A19, 430K10, 487A02, 95C14, 302C13, 66G15 and 266K03) produced strong signals as homogeneous staining regions (HSRs) (Fig. 1a, b), indicating that they each represented part of the amplicon. In contrast, the other five BACs failed to show HSR patterns in the same cell line, reflecting positions outside the amplicon (Fig. 1b). In RAMOS cells, 15 BACs produced five signals and two BACs each produced two signals (Fig. 1b). In OCI-LY3 and OCI-LY1, all 17 BACs produced four and three signals, respectively (Fig. 1a, b). Taking these findings together, we defined the smallest region of overlap (SRO) between BACs 321E11 and 266K03. We estimated its extent to be 4 Mb, on the basis of the genome database archived by the UCSC. According to the NCBI database, this region harbors the *GPC5* gene and 15 uncharacterized, possibly transcribed sequences (Table 1).

Expression of *GPC5* gene in lymphoma cell lines

As Table 1 indicates, the sequences and genomic structures of the database-predicted transcripts, as well as the RT-PCR results followed by direct sequencing and northern blotting (data not shown), demonstrated that none of the 16 predicted transcripts except *GPC5* were true genes. For example, no expressed-sequence tags (ESTs) or evidence of exons had been filed in genome databases, and no intronic sequences were detected, indicating that the predicted transcripts were probably pseudogenes; moreover, no specific RT-PCR products with correct sequences were detected for any of the 15 predicted sequences, even though we used more than two primer sets for each of them (data not shown). On the basis of the database-derived and experimental evidence, we concluded that none of the 15 predicted sequences was a true gene, and that the 4-Mb SRO contained only one actual gene, *GPC5*. Therefore we focused on *GPC5* as a potential target for amplification at 13q31-q32.

As a second step toward determining whether *GPC5* was in fact the target for amplification in our SRO, we evaluated whether this gene was consistently overexpressed as a consequence of gains in copy number (Imoto et al. 2001). All six lymphoma cell lines showed detectable expression of *GPC5*, although the relative expression levels in cell lines with increased copy numbers of *GPC5* were consistently higher than in the lines with only two copies (Fig. 1c). Those data suggested that this gene might indeed be activated by the amplification mechanism and represent an actual target of amplification in lymphoma cell lines. However, *GPC5* expression levels did not always parallel the copy numbers, indicating that other genetic and/or epigenetic mechanisms may contribute to increased expression of this gene.

GPC5 is a member of the glypican family, a group of membrane-bound heparan sulfate proteoglycans (HSPGs) that are linked to the cell surface by a glycosylphosphatidylinositol (GPI) anchor (Filmus and Song 2000). Six members of this molecular family

Table 1 Summary of one known gene and 15 uncharacterized, predicted transcripts within the SRO of the 13q31-q32 amplicon detected in lymphoma cell lines

Symbol	GenBank accession number	Functional suggestions or other comments[a]	Evidence for not a true gene		
			Evidence of ESTs[b]	Genomic structure[c]	RT-PCR[d]
LOC121723	XM_062763	Similar to mt-GrpE protein homologue 2	None	N	F
LOC160824	XM_090522	Similar to Sp3 (SPR-3)	3[e]	N	F
LOC121725	XM_062764	Similar to Hsp70	4[e]	N	F
LOC204113	XM_118427	Similar to ATF4	1	N	F
LOC144774	XM_090523	Similar to ribosomal protein L7A	None	N	F
LOC121727	XM_062765	Similar to PAF-3 [Peroin-12 (PEX12)]	None	P	F
LOC121728	XM_062766	Similar to MALE STERILITY PROTEIN 2-LIKE PROTEIN	None	P	F
LOC121729	XM_062767	Similar to cytokeratin 18	1	P	F
LOC144776	XM_084964	Similar to rad6, itchy	none	N	F
LOC160826	XM_101250	None	2[e]	4 predicted exons	F
LOC121734	XM_062758	Similar to cyclophilin-type peptidyl-prolyl *cis-trans* isomerase	1	P	F
LOC160827	XM_101252	None	23[e]	2 predicted exons	F
GPC5	XM_012278	Glypican 5		Known gene	
LOC204114	XM_115201	Similar to fatty acid-binding protein	18[e]	P	F
LOC160830	XM_101254	None	7[e]	N	F
LOC160906	XM_090580	Similar to glypican-5 precursor	10[e]	N	F

[a]Functional suggestions or other comments listed in LocusLink (http://www.ncbi.nlm.nih.gov/LocusLink)
[b]ESTs listed in LocusLink
[c]Genomic structure and sequence described in LocusLink (Evidence Viewer); *N* no evidence of exonic sequence, *P* probable pseudogene
[d]RT-PCRproduct was evaluated with regular electrophoresis, direct sequence, and Northern blotting; *F* failed to detect specific RT-PCR product with correct sequence
[e]ESTs are listed but not matched to the predicted sequence by BLAST (http://www.ncbi.nlm.nih.gov/BLAST/)

(*GPC1–GPC6*) have been identified in mammals (Filmus et al. 1988; David et al. 1990; Stipp et al. 1994; Watanabe et al. 1995; Veugelers et al. 1997; Saunders et al. 1997; Veugelers et al. 1999; Paine-Saunders et al. 1999). In general, glypicans are expressed predominantly during development and their expression levels change in a stage- and tissue-specific manner, suggesting that glypicans are involved in the regulation of morphogenesis (Litwack et al. 1994; Saunders et al. 1997; Pellegrini et al. 1998). Since glypicans can regulate the activity of growth and survival factors, changes in their expression could very well be associated with tumor progression. For example, *GPC1* expression was significantly increased in a large proportion of pancreatic cancers (Kleef et al. 1998). *GPC3* expression was down-regulated in a significant proportion of ovarian-cancer cell lines (Lin et al. 1999) and in mesothelioma cell lines (Murthy et al. 2000), but up-regulated in most hepatocellular carcinomas (Hsu et al. 1997) and a significant proportion of colorectal tumors (Filmus and Selleck 2001).

As *GPC5* is expressed in various embryonic tissues but only in brain tissue in the adult, *GPC5* expression may have a general role in the control of growth and differentiation during mammalian development (Veugelers et al. 1997). In Northern blotting experiments most cultured tumor-cell lines, including acute lymphoblastic leukemia, histiocytic lymphoma, and myeloma, have been negative for *GPC5* (Veugelers et al. 1997). However, the expression patterns reported here, as well as functions of other members of the glypican family, strongly suggest that altered expression of *GPC5*, especially over-expression through gene amplification, might play an important role in the pathogenesis of lymphomas. Further studies will be necessary to evaluate the amplification and expression status of this gene in primary cases of lymphoma and to determine the functional roles of GPC5 in the pathogenesis of these tumors.

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