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Research article

CLONING AND AMPLIFICATION OF ZNF 217 GENE IN CANCER

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ABSTRACT: ZNF 217 been increasingly recognized that znf 217 in human cancers may be complex structures composed of large amplified DNA domains containing multiple genes. It is therefore important to devise strategies for the rapid isolation of cDNAs expressed from these structures. Using a procedure we term microdissection mediated cDNA capture, we recovered znf 217 specific cDNAs from two different human tumors. The glioblastoma cell line TX3868 and the human sarcoma cell line OsA-CL carry ZNF 217 containing amplified sequences from chromosome 12q13–15. We recovered 2 specific cDNAs following microdissection of this ZNF 217 which had been previously hybridized in situ with linkered cDNA. Northern blot analysis with these cDNAs revealed hybridization to distinct transcripts in OsA-CL RNA and TX3868 RNA. None of the OsA-CL cDNA clones showed cross hybridization with the TX3868 cDNAs suggesting that despite their coincident band localization on 12q, the OsA-CL and TX3868 amplification units do not completely overlap. These results significantly increase the number of amplified genes assigned to the 12q13–15 amplicon illustrating both the complexity of ZNF 217 derived from this region and the utility of microdissection mediated cDNA capture to gain rapid access to cDNAs transcribed from amplified genes.
Key words: Cloning, Amplification, ZNF 217 gene, Cancer

INTRODUCTION

Cancer is a group of diseases in which cells are aggressive (grow and divide without respect to normal limits), invasive (invade and destroy adjacent tissues), and sometimes metastatic (spread to other locations in the body) [1-5]. These three malignant properties of cancers differentiate them from benign tumors, which are self-limited in their growth and don't invade or metastasize (although some benign tumor types are capable of becoming malignant) [6-10]. All cancers begin in cells, the body's basic unit of life [11]. The body is made up of many types of cells. These cells grow and divide in a controlled way to produce more cells as they are needed to keep the body healthy. When cells become old or damaged, they die and are replaced with new cells. But sometimes this orderly process goes wrong [12-16]. The genetic material (DNA) of a cell can become damaged or changed, producing mutations that affect normal cell growth and division [17]. When this happens, cells do not die when they should and new cells form when the body does not need them. The extra cells may form a mass of tissue called a tumor. These growths are considered either benign or malignant. Benign is considered non-cancerous and malignant is cancerous [18-22]. Cancer may affect people at all ages, even fetuses, but risk for the more common varieties tends to increase with age. Cancer causes about 13% of all deaths. According to the American cancer society (ACS), 7.6 million people died from cancer in the world during 2007 [23, 24]. The cancer is caused by the chemical carcinogens, infectious diseases, ionizing radiation, hormonal imbalances, immune system dysfunction heredity etc. The signs and symptoms of the cancer are Lack or loss of appetite (Anorexia), unusual lumps or swelling (tumor), hemorrhage (bleeding), indigestion or difficulty swallowing , non-healing sores,headaches, Back pain, pelvic pain, bloating, or indigestion,unexpected weight loss, night sweats, or fever [25].

MATERIALS AND METHODS

Cell culture and metaphase preparations

Cell culture and cytogenetic studies were performed and tumor samples were stored in liquid nitrogen immediately after surgical removal. Following xenografting of tumor cells from the glioblastoma cell line T3868 into nude mice, the glioblastoma cell line TX3868 was established.

RNA isolation, cDNA synthesis, and linker addition (OsA-CL)

RNA was isolated from cultured tumor cells by the single step guanidium thiocyanate method. Fifteen mg of oligo dT selected mRNA was used for random primed cDNA synthesis (Riboclone cDNA synthesis kit, Promega). Following second strand synthesis, the cDNA was phenol: chloroform extracted and ethanol precipitated. The cDNA was digested with 50 U *Xho*I for 3 h and then made blunt ended with T4 polymerase as indicated in the manufacturer's protocol. The cDNA was modified for PCR amplification. Two mg blunt ended cDNA were ligated to 15 mg of phosphorylated catch A (54GAGTAGAATTCTAATATCTC 34) and 15 mg of phosphorylated Catch B (54GAGATATTAGAATTCTACTC 34) linkers for 48 h at 16_C in a volume of 40 ml. After ligation, the cDNA was digested overnight with 100 U *Xho*1 to remove linker concatemers and purified over two successive Sephadex G-50 columns. One fifth (10 ml) of the ligated, digested cDNA was amplified in a 50 ml reaction containing 7.5 U Taq polymerase (Perkin-Elmer Cetus), 2.5 mM catch A primer, 200 mM of each dNTP, 50 mM KCl, 1.5 mM MgCl2, and 10 mM Tris–HCl, pH 8.4. The cDNA was denatured for 30 min at 94_C in the presence of primer and buffer. The dNTPs and Taq polymerase were added for 15 cycles of 94_C 1 min, 40_C 1 min, 72_C 1 min, and a final extension of 72_C 5 min. The cDNA was digested overnight with 50 U *Xho*1 and purified over two successive Sephadex G-50 columns. Five ml (1/10) of the cDNA was amplified for 20 cycles in a 50 ml PCR as described above but with an annealing temperature of 52_C. The product of this reaction is the stock cDNA used for further PCR and for biotin labeling. All subsequent amplifications of cDNA destined for hybridizations used 1 ml of stock cDNA for a maximum of 20 cycles with 0.25 mM of catch A. Biotin labeling was done under the same conditions but with 130 mM dTTP and 150 mM biotin-16-dUTP.

RNA isolation, cDNA synthesis, and linker addition (TX3868)

RNA was isolated from cultured tumor cells according to Gough and digested with RNase free DNase (Boehringer Mannheim) for 15 min at 37_C. After ethanol precipitation 9 mg RNA were used for cDNA synthesis. cDNA synthesis was primed with an oligo d(T)15 primer following the manufacturers protocol (Boehringer Mannheim). Two ng blunt ended cDNA were ligated to 2 pmoles of Uni-ampTM Adaptor/*Sal*I for 20 h at 16_C according to the Uni-ampTM Adaptor ligation protocol (Clontech) in a volume of 10 ml. One third of the ligated cDNA was amplified in a 50 ml reaction containing 2.5 U Taq Polymerase, 25 mM Uni-Amp Primer (Clontech), 200 mM of each dNTP and 1.5 mM MgCl2. The reaction was first denatured for 3 min at 94_C following 30 cycles of 94_C for 1 min, 60_C for 1 min, 72_C for 2 min and a final extension step at 72_C for 10 min. In a secondary PCR, 1 ml cDNA PCR product was labeled with biotin-16-dUTP. The PCR conditions were as described above except 1 ml of biotin-16-dUTP (1 mM) was added.

cDNA *in situ* hybridization

For OsA-CL hybridizations, cDNAs (5 mg) were added to a 10 ml hybridization mix that included 5 mg of Cot1 DNA (BRL), 19 mg of herring sperm DNA, 5% dextran sulfate, 1× SSC, 1× SSPE, and 1% Tween 20. The hybridization mix was added to 1 week old untreated slides, sealed with rubber cement and placed in a moist chamber for 20 min. The chromosomes and cDNAs were denatured *in situ* for 10 min at 100_C in a steam bath inside a covered beaker of boiling water. Hybridization was carried out for 18 h at 66_C in a moist chamber inside a water bath. For biotin labeled hybridizations slides were washed to a stringency of 2× SSC at 45_C. TX3868 hybridizations were done according to the method of Hozier. Fluorescent detection of the hybridized probe followed the method of Pinkel. Slides hybridized with unlabeled cDNA were washed to a stringency of 0.1× SSC, 0.05% Tween 20 at 65_C. These slides were stained with Giemsa for 10 min prior to microdissection.

Microdissection

Microdissection of the cDNA-hybridized hsrs was performed using a glass needle controlled by a hydraulic micromanipulator (Narashige) as previously described. For amplification of genomic DNA, seven copies of an hsr were microdissected and transferred to a 5 ml collection drop containing 200 mM of each dNTP, 1 mM UN1 primer (CCGACTCGAGNNNNNNATGTGG) and 0.2 U topoisomerase I (Promega). After microdissection the collection drop was covered with a drop of mineral oil and incubated at 37_C for 30 min. cDNA-hybridized hsrs were microdissected and transferred to a 5 ml collection drop containing 200 mM of each dNTP, 1mM catch A primer (or 0.5 mM Uni-AmpTM Primer), 0.25 U Taq polymerase, 50 mM KCl, 1.5 mM MgCl2, and 10 mM Tris–HCl, pH 8.4. After microdissection the collection drop was covered with a drop of mineral oil prior to PCR.

Amplification of micro dissected cDNAs

For OsA-Cl microdissections, the 5 ml collection drop with the microdissection material was pre-amplified for 15 cycles of 1 min 94_C, 1 min 52_C and 1 min 72_C. The volume was increased to 60 ml with 1.25 U Taq polymerase, 200 mM of each dNTP, 0.25 mM catch A and amplified for 20 more cycles. A 10 ml aliquot from the first PCR was used in a 50 ml second round PCR for 30 more cycles as described but with 2.5 U Taq polymerase and 0.5 mM catch A. For TX3868 microdissections, the dissected hsrs and bound cDNAs were transferred to a PCR tube and amplified by 50 cycles of PCR (1 min at 94_C, 1 min at 60_C and 2 min at 72_C) with 2.5 U Taq polymerase.

Cloning of PCR products

The microdissection selected cDNAs from OsA-CL were prepared for UDG cloning. Four ml of cDNA were amplified for 20 cycles using CUA catch A primer (54CUACUACUACUAGAGTAGAATTCTAATATCTC 34) under the PCR conditions for stock cDNA amplification described above. The PCR products were purified with a QUIquick-spin column (Quiagen) and ethanol precipitated. Fifty ng of cDNA were mixed with 50 ng of UAGpUC19 (pUC19 PCR amplified for UDG cloning with UAG containing primers), incubated with uracil DNA glycosylase, and transformed into DH5a cells according to the manufacturer's protocol (GibcoBRL). 288 randomly chosen colonies were arrayed in 96 well plates for replica plating, long term storage at –80_C and for insert isolation through 30 cycle PCR with catch A primer.

The selected cDNAs from TX3868 were purified with PrimerEraseQuick (Stratagene), phosphorylated, self-ligated, digested with *Sal*I, ligated into pBluescript and transformed in DH5 a cells as described by Klein *et al*.

Southern and Northern blot analysis

Isolation of high molecular weight DNA from cell cultures was performed according to standard protocols. Southern and Northern hybridization was carried out according to standard protocols. For the amplification and expression studies, 50 ng clone insert generated by PCR with catch A primer was labeled with 32P by the random priming method. Human multiple tissue Northern blots were obtained from Clontech.

RESULTS AND DISCUSSION

After running the PCR, we got the product was obtained near 400bp region. That is, the fragment size after amplification is found to be 400bp. Since, this is a partial clone; efforts are underway to pull out the full length clone.

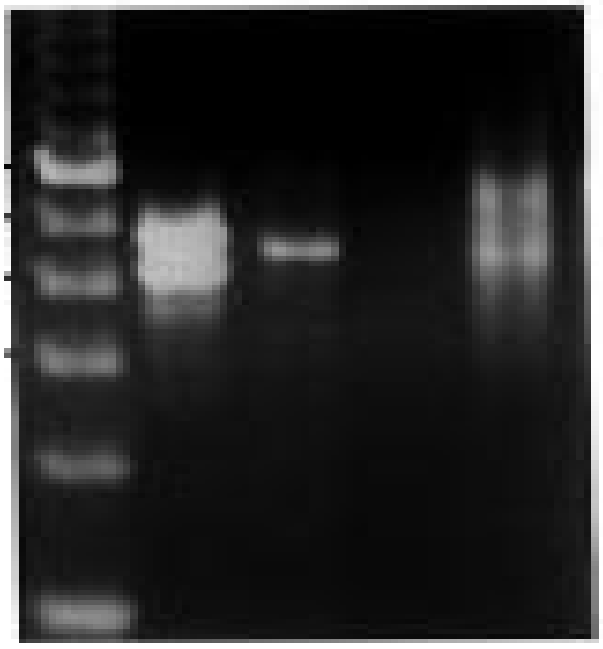

Fig 1: Agarose gel electrophoresis of recovered cDNA

The ZNF 217 gene was amplified and agarose gel electrophoresis method was used to find the gene length. The cell lines used for isolation of ZNF 217 were tabulated in Table 1.

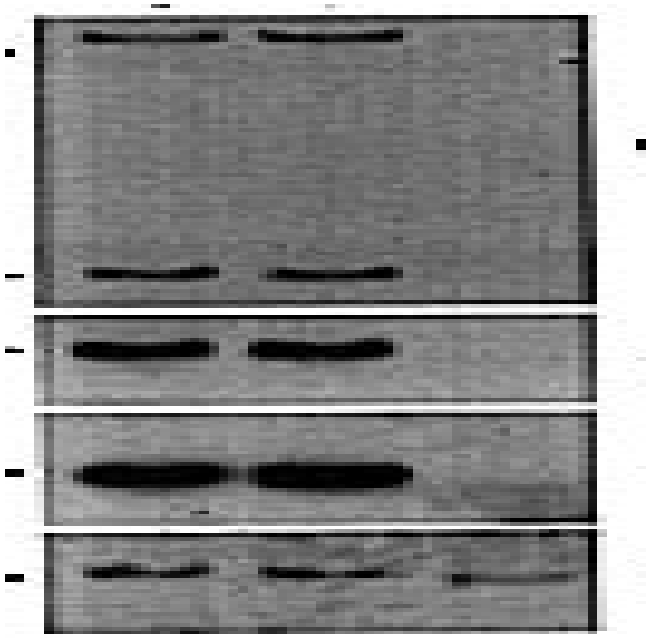

Fig 2: Amplification of ZNF 217 into the cell lines

Table 1: OsA, NGP and RMS are the cell lines from which ZNF 217 is isolated

Number of clones	Amplification status[a]			mRNA size (kb)[b]	Insert size (bp)[c]
	OsA	NGP	RMS		
2	+	+	–	5.5	306
5	+	+	+	4.8	399

The gene expression studies were studied using Northern blotting technique.

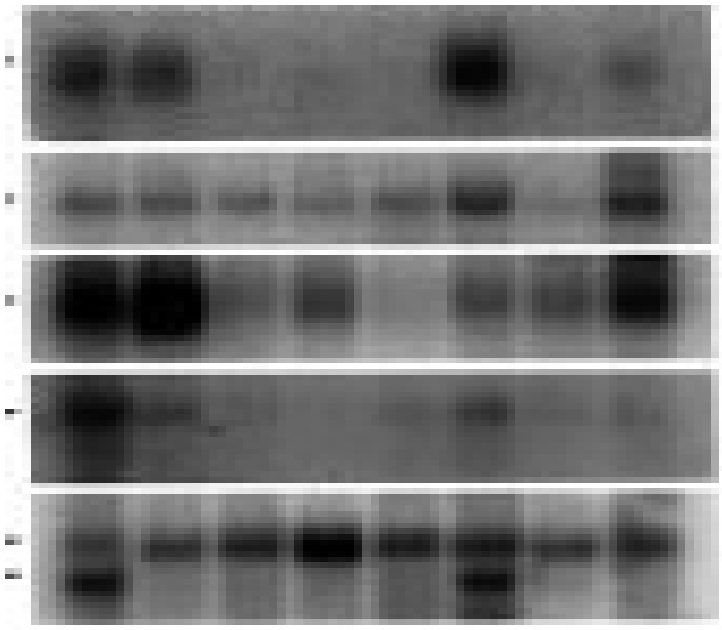

Fig 3: Northern blotting results

CONCLUSION

It is of considerable interest to identify the amplified genes found in ZNF 217 in order to assess the impact of their expression on tumor phenotype. However, cDNAs for amplified genes are difficult to clone without previously mapping the amplification unit at the genomic level. In this report, we demonstrate the utility of microdissection mediated cDNA capture for the isolation of transcribed sequences encoded in an ZNF 217. Using the strategy presented above, we have now been successful in using microdissection mediated cDNA capture to isolate transcripts from two different ZNF 217. The cDNA libraries generated following microdissection were highly enriched for sequences derived from the amplification unit. Because this approach combines the use of cDNA from the ZNF 217 bearing cell line with exploitation of the genomic composition of the hsr itself, it avoids false positives such as retro-pseudogenes which might confound alternative strategies such as direct hybridization of cDNA libraries with microdissected hsr sequences. Thesensitivity of this procedure is more difficult to assess. Our results strongly suggest that microdissection mediated cDNA capture is a useful approach for the isolation of cDNAs encoded by amplified genes. ZNF 217 is one of the Zinc finger regulator proteins, which specifically binds with the caspase 8 and caspase 10.

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