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Retroviral expression screening of oncogenes in pancreatic ductal carcinoma

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Abstract

Pancreatic ductal carcinoma (PDC) remains one of the most intractable malignancies in humans. In order to clarify the molecular events underlying the carcinogenesis in PDC, we constructed a retroviral cDNA expression library from a PDC cell line, and used it to screen transforming genes in PDC by a focus formation assay with mouse 3T3 fibroblasts. We could obtain a total of 30 transformed cell foci in the screening, and one of the cDNA inserts harvested from such cell clones turned out to encode a wild-type human ARAF1. Unexpectedly, a long terminal repeat-driven overexpression of *ARAF1* mRNA was confirmed to induce transformed foci in fibroblasts. The oncogenic potential of ARAF1 was examined by injecting the transformed fibroblasts into athymic nude mice. Importantly, *ARAF1* mRNA was highly expressed in pancreatic ductal cell specimens purified from patients with PDC. These results have unveiled the transforming potential of ARAF1 protein, and also suggest that quantity of intracellular ARAF1 may be important in carcinogenesis of various human cancers.

Keywords: Pancreatic ductal cell carcinoma; Retrovirus; ARAF1; cDNA library; Oncogene

1. Introduction

Pancreatic ductal carcinoma (PDC) originates from pancreatic ductal cells, and is one of the most intractable malignancies in humans [1,2]. Effective therapy for PDC is hampered by the lack of specific clinical symptoms. At the time of diagnosis, most patients are no longer candidates for surgical resection, and, even in individuals who do undergo such surgery, the 5-year survival rate is only 20–30% [1].

Vast efforts have been made to elucidate molecular events responsible for the carcinogenesis of PDC. Mutations of *TP53* gene can be, for instance, found in PDC specimens [3], and in the intraductal *in situ* regions as well [4]. Similarly, inactivation has been found for other tumour-suppressor genes, such as DPC, RB1 and p16 [5].

As for oncogenes, activating mutations in the *KRAS2* gene has been reported to be frequently associated with PDC [6]. The same *KRAS2* mutations could be, however, found in the samples for chronic pancreatitis [7], making their pathogenetic role uncertain. Additionally, an increased telomerase activity was shown to be present only in PDC, but not in nonmalignant pancreatic disorders [8]. Again, however, others could detect an elevated telomerase activity in chronic pancreatitis and normal

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pancreas [9]. Therefore, it is yet to be revealed which transforming genes truly promote a clonal growth of pancreatic ductal cells.

For an efficient isolation of tumour-promoting genes in PDC, it would be desirable to conduct functional screening based on transforming ability. Focus formation assays with mouse 3T3 fibroblasts have been highly successful for the identification of oncogenes in human cancer [10]. In such screening, genomic DNA is isolated from cancer specimens, and used to transfect 3T3 cells to obtain transformed cell foci. It should be noted, however, that, since expression of any genes in these experiments are driven by their own promoters/enhancers, oncogenes in PDC can exert their effects in 3T3 cells only when the promoter/enhancer regions of such genes are active in fibroblasts, which is not always guaranteed.

To ensure the sufficient expression of cDNAs in 3T3 cells, their transcription should be regulated by an exogenous promoter fragment. We have therefore constructed a retroviral cDNA expression library from a PDC cell line, MiaPaCa-2, which was used to infect 3T3 cells. In the preparation of cDNA library, we further took advantage of the SMART polymerase chain reaction (PCR) system (Clontech, Palo Alto, CA), which preferentially amplifies full-length cDNAs. A focus formation assay with the library resulted in an identification of a transforming *ARAF1* gene.

2. Materials and methods

2.1. Cells and culture

MiaPaCa-2, Capan-2, PANC-1, 3T3, and BOSC23 [11] cell lines were obtained from American Type Culture Collection, and maintained in Dulbecco's modified Eagle medium/F12 (DMEM/F12; Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Invitrogen) and 2 mM L-glutamine.

The fresh clinical specimens were obtained from patients who gave informed consent, and the study was approved by the institutional review board of Jichi Medical School. Cells were collected from the pancreatic juice by centrifugation, labeled with anti-MUC1 antibody [12] (Novocastra Laboratories, Newcastle upon Tyne, UK), and subjected to chromatography on a miniMACS magnetic cell separation column (Miltenyi Biotec, Auburn, CA) [13]. The purity of the resultant $MUC1^{+}$ cell fraction was confirmed by staining with Wright Giemmsa solutions and microscopy examination for each case (data not shown).

2.2. Retrovirus library construction

Total RNA was extracted from MiaPaCa-2 cells by an RNeasy Mini column with RNase-free DNase (Qiagen, Valencia, CA), and the first strand cDNA was synthesized by PowerScript reverse transcriptase (Clontech) with SMART IIA oligonucleotide and CDS primer IIA (both from Clontech). The cDNAs were then amplified for 12 cycles with 5′ PCR primer IIA according to the instruction of the SMART PCR cDNA synthesis kit (Clontech) except a substitution of LA Taq polymerase (Takara Bio, Shiga, Japan) for Advantage 2 DNA polymerase provided with the kit. Resultant cDNAs were treated with proteinase K, blunt-ended by T4 DNA polymerase, and ligated to the BstXI-adaptor (Invitrogen). Unbound adaptors were removed through the cDNA size fractionation column (Invitrogen), and the cDNAs were finally ligated to the pMXS retroviral plasmid (a kind gift of Dr. T. Kitamura at Institute of Medical Science, University of Tokyo) [14] digested with BstXI. The pMXS-cDNA plasmids were introduced into ElecroMax DH10B cells (Invitrogen) with electroporation.

2.3. Focus formation assay

Generation of recombinant retroviral library and focus formation assay was conducted as described previously [15]. Briefly, BOSC23 cells were transfected with Lipfectamin reagent (Invitrogen) and 2 μg of retroviral plasmid together with 0.5 μg of pGP plasmid 0.5 μg of pE-eco plasmid (both from Takara Bio). Two days after the transfection, polybrene (Sigma, St. Louis, MI) was added to the culture supernatant at a concentration of 4 μg/ml, and the supernatant was subsequently used to infect 3T3 cells for 48 h. For the focus formation assay, the culture medium of 3T3 cells was then changed to DMEM-high glucose (Invitrogen) supplemented with 5% calf serum and 2 mM L-glutamine. Transformed foci were picked up after 3 weeks of culture. Genomic DNA was extracted from each transformed focus, and was subjected to PCR with 5′ PCR primer IIA (Clontech) and LA Taq polymerase for 50 cycles of 98 °C for 20 s and 68 °C for 6 min. Amplified genome fragments were purified for nucleotide sequencing. For tumorgenicity assay in nude mice, transformed 3T3 cells were injected into each shoulder of nu/nu Balb-c mice (6 weeks old). Tumour formation was assessed after 4 weeks.

2.4. "Real-time" RT-PCR

Portions of oligo(dT)-primed cDNA were subjected to PCR with a QuantiTect SYBR Green PCR Kit (Qiagen). The amplification protocol was comprised of incubations at 94 °C for 15 s, 57 °C for 30 s, and 72 °C for 60 s. Incorporation of the SYBR Green dye into PCR products was monitored in real time with an ABI PRISM 7900HT sequence detection system (PE Applied Biosystems, Foster City, CA), thereby allowing determination of the threshold cycle (C_T) at which exponential amplification of products begins. The C_T values for cDNAs corresponding to the β-actin gene (*ACTB*) and to the *ARAF1* gene were used to calculate the abundance of the latter mRNA relative to that of the former. The oligonucleotide primers for PCR were as follows: 5′-CCATCATGAAGTGTGACGTGG-3′ and 5′-GT-CCGCCTAGAAGCATTTGCG-3′ for *ACTB*, and 5′-ACTACCTCCATGCCAAGAACATCA-3′ and 5′-GACGTCTGACTGGAAGCTGTAGGG-3′ for *ARAF1*.

3. Results

3.1. Screening with focus formation assay

From mRNA of MiaPaCa-2 cells, full-length cDNAs were selectively amplified and ligated to a retroviral vector pMXS. We could obtain a total of 2.1×10^6 colony forming units (cfu) of independent plasmid clones. Thirty clones were randomly selected from the library, and examined for the incorporated cDNAs. Twenty-seven (90%) out of the 30 clones contained inserts with an average length of 2.05 kbp.

By introducing the plasmid DNA into a packaging cell line, we generated a recombinant ecotropic retrovirus library that was subsequently used to infect mouse 3T3 fibroblasts. Infection experiments were repeated for a total of 6 times. After 3 weeks of culture, 30 transformed foci were observed (Fig. 1(b)). No foci could be found among the cells infected with an empty virus (Fig. 1(a)), while numerous foci were easily identified in the cells infected with a virus expressing v-Ras oncoprotein (Fig. 1(c)).

Each focus was isolated, expanded independently, and was subject to the extraction of genomic DNA. We then tried to recover retroviral inserts from the genomic DNA by PCR amplification with the primer used originally to amplify the cDNAs in the construction of the library. In most cases, two to three DNA fragments were recovered from each genome (Fig. 2(a)), implying a multiple retroviral infection on the recipient 3T3 cells.

We obtained a total of 56 cDNA fragments by PCR, all of which were subjected to nucleotide sequencing from both ends. Screening of the cDNA sequences against human genome sequence database assembled as of July 2003 by the Genome Bioinformatics Group of the University of California at Santa Cruz (http://genome.ucsc.edu) revealed that the 56 fragments correspond to 13 independent genes, eleven of which could be matched at >95% identity to the human genome sequence (Table 1). Among the 11 genes, 7 of them were known genes while the rest 4 were unknown. Each cDNA clone was ligated to pMXS in both directions, and a recombinant retrovirus was generated from each resultant plasmid. Transforming ability of the cDNAs was thus confirmed by a focus formation assay with the recombinant virus.

Focus formation assays were conducted for the 26 independent viruses (all 13 independent genes for both directions), and one of them, expressing ARAF1 protein (GenBank accession number, NM_001654), gave transformed foci in repeated experiments. ARAF1 belongs to

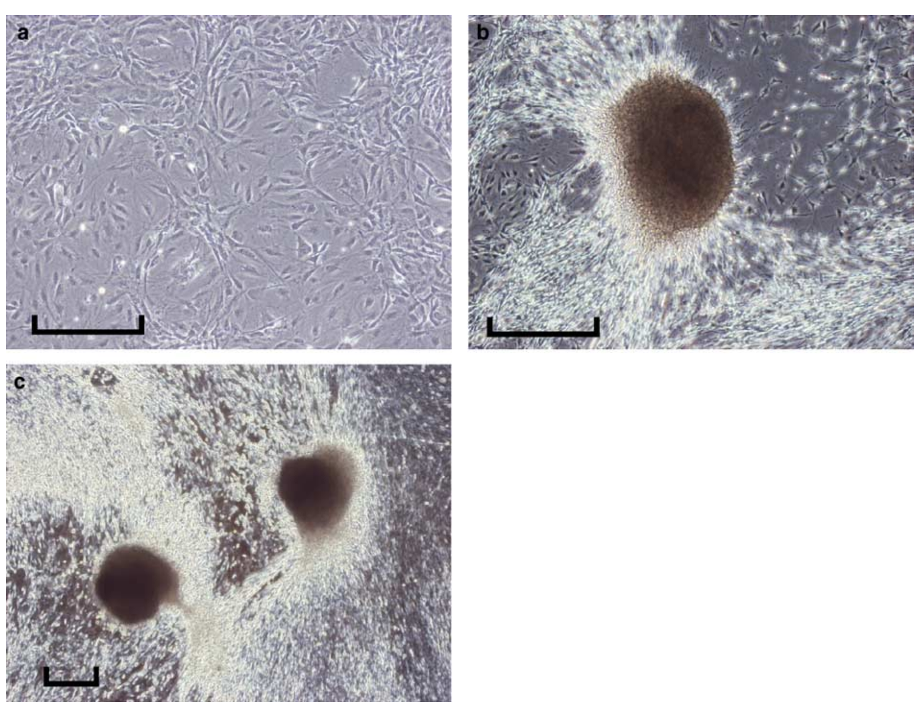

Fig. 1. Focus formation assay with retroviral library. Mouse 3T3 cells were infected with (a) an empty virus, (b) retroviruses from the MiaPaCa-2 library, or (c) a retrovirus expressing v-Ras as a positive control. Pictures were taken after 3 weeks of culture. Scale bar, 100 μm.

Table 1
cDNAs isolated from 3T3 transformants

Clone #	Gene symbol	GenBank number	Covering full ORF
1	Unknown	AK026325	ND
2	No homologues sequences	ND	ND
3	Unknown	AF318370	Yes
4	PITPNM1	NM_004910	No
5	Unknown	BC022099	Yes
6	DECR2	NM_020664	Yes
7	ARAF1	NM_001654	Yes
8	BCLG	NM_138724	No
9	No homologues sequences	ND	ND
10	Unknown	AL607122	ND
11	JAG1	NM_000214	Yes
12	MRPL43	NM_176792	Yes
13	PLOD3	NM_001084	No

ORF, open reading frame; ND, not determined.

the RAF family of serine/threonine kinases, and phosphorylates MEK1 [16]. It had not been known whether an overexpression of wild ARAF1 protein has a transforming activity.

3.2. ARAF1 as an oncogene

We thus determined the whole nucleotide sequence of our *ARAF1* cDNA (cDNA clone ID #7). The sequence is 2441 bp, and contains an open reading frame (spanning nucleotide position 126–1943) encoding a protein of 606 amino acids, which is identical to ARAF1 (Fig. 2(b)). Within the protein-coding region, there is only one nucleotide mutation compared to the published *ARAF1* cDNA sequence; a "T" at nucleotide position 1550 in the reported sequence (NM_001654) is replaced with a "C" in our sequence. The codon sequence "TTG" at the amino acid position 450 of ARAF1 is thus changed to "CTG" in our cDNA. However, both codons encode the same leucine residue, and thus the mutation does not affect the protein sequence.

To confirm that mere overexpression of wild ARAF1 protein has a transforming activity, we repeated the focus formation assay with the retrovirus generated from our *ARAF1* cDNA. As shown in Fig. 2(c), the recombinant virus reproducibly induced transformed foci (30–50 foci per microgram of the input plasmid) in the recipient 3T3 cells. The transforming ability of ARAF1 was also tested by the tumorgenicity assay with athymic nude mice. The 3T3 cells infected with the empty virus or retrovirus expressing ARAF1 or v-Ras were inoculated subcutaneously into nude mice. As shown in Fig. 2(d), tumour formation was observed in all mice for the latter 2 cases, arguing that ARAF1 has oncogenic potential.

3.3. Expression of ARAF1 in PDC

We finally measured the expression level of *ARAF1* mRNA in PDC by the quantitative real-time reverse

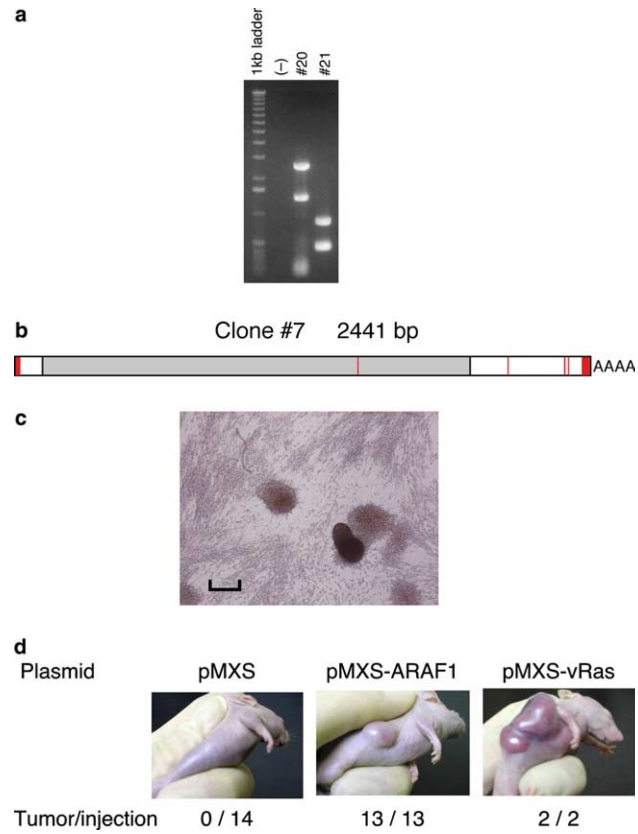

Fig. 2. Identification of the transforming *ARAF1* gene. (a) Genomic DNAs isolated from the transformed foci (cell clone ID #20 and #21) were subjected to PCR. A PCR without DNA template was also conducted as a negative control (–). DNA size markers (1 kb DNA ladder; Invitrogen) are electrophoresed at the left. (b) A 3T3 clone yielded a PCR product of 2441 bp long (cDNA clone ID #7). The cDNA has a protein-coding region (gray) for 606 amino acids that was identical to human ARAF1 protein. Nucleotides that did not match the published *ARAF1* cDNA are indicated by red lines. (c) Our ARAF1 cDNA was ligated to pMXS, and used to generate recombinant virus. Infection with the virus induced multiple transformed foci in 3T3 cells. Scale bar, 100 μm. (d) 3T3 cells (5×10^5) were cultured for two days with retrovirus made from pMXS, pMXS-ARAF1 or pMXS-vRas plasmid, and were injected subcutaneously into nu/nu mice. Tumour formation was examined after 4 weeks.

transcription (RT)-PCR method. As shown in Fig. 3, all 3 PDC cell lines express similar amounts of *ARAF1* mRNA. In addition, we quantified *ARAF1* mRNA in human clinical specimens. Pancreatic juice from patients with PDC contains cancer cells (transformed ductal cells) in addition to normal ductal cells and blood cells. The former two fractions were purified, by an affinity column for MUC1 surface protein [12], from pancreatic juice of PDC patients ($n = 14$). Such purified fractions should be highly enriched for PDC cells [13]. Similar MUC1-positive fractions were also purified from the pancreatic juice of healthy individuals ($n = 7$). Quantification of *ARAF1* mRNA revealed that its mRNA level was highly elevated in six out of the 14 patient, but not in the specimens from healthy individuals. These data

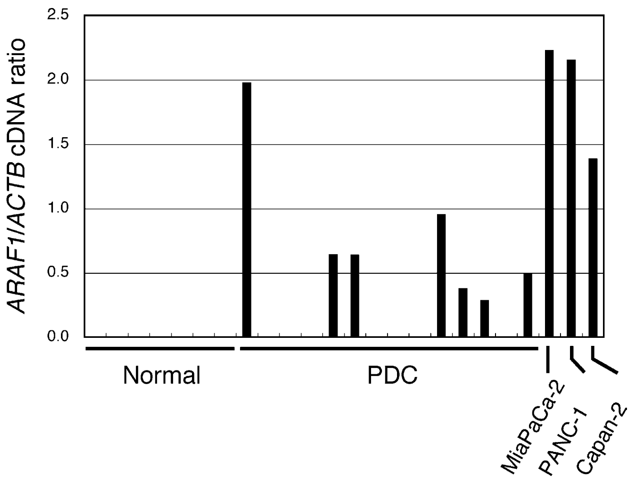

Fig. 3. Quantification of *ARAF1* mRNA. Complementary DNA was prepared from PDC cell lines (MiaPaCa-2, PANC-1 and Capan-2) or MUC1$^+$ ductal cell preparations purified from healthy individuals (Normal) or patients with PDC, and were then subjected to real-time RT-PCR analysis with primers specific for the *ARAF1* or *ACTB* genes. The ratio of the abundance of *ARAF1* mRNA to that of *ACTB* mRNA was calculated as 2^n, where n is the C_T value for *ACTB* cDNA minus the C_T value for *ARAF1* cDNA.

indicate that the transcription of *ARAF1* is selectively activated in PDC cells.

4. Discussion

In this manuscript, we have constructed a retroviral cDNA expression library of PDC. Since 90% (27/30) of the viral plasmids carried cDNA inserts and since the overall clone number was >2 millions, our library should cover nearly all transcriptome in MiaPaCa-2 cells.

RAF family is composed of RAF1, ARAF1 and BRAF in humans. All these serine/threonine kinases are believed to act downstream of RAS-family proteins, and to phosphorylate and regulate downstream MAP kinase kinases (MAPKKs). Many studies have revealed transforming potentials of RAF family proteins. RAF was originally identified as a cellular homologue of viral oncoprotein, v-Raf [17]. Deletion of amino-terminal regions unmasks the transforming ability of RAF1 [18] and ARAF1 [19]. On the other hand, somatic point mutations have been found in the *BRAF* gene among clinical specimens of colorectal carcinoma [20]. Such mutations were shown to induce transforming activity in BRAF protein. In contrast to *BRAF*, somatic point mutations are rarely found in *ARAF1* gene [21].

Overexpression of wild forms of RAF1 or BRAF failed to exert a transforming activity [18,20]. Although deletion/truncation of amino terminal regions of ARAF1 induced transformed foci in 3T3 fibroblasts [22] and abrogated cytokine-dependency in hematopoietic cells [19], it has not been tested whether wild ARAF1 protein has transforming potential. In this manuscript, however, it was unexpectedly revealed that a long terminal repeat-driven expression of ARAF1 induces transformed foci in 3T3 cells, which subsequently generated tumours in immunocompromised mice. Therefore, it has been unveiled here that an overexpression of wild ARAF1 is directly linked with cellular transformation process.

These data also indicate the importance of measuring protein/mRNA amounts of ARAF1 in various human cancers. In this context, it was interesting to find a high expression of *ARAF1* mRNA in fresh clinical specimens of PDC. Our findings shed new light on the understanding of RAF family kinases, and open up the possibility that ARAF is involved in carcinogenesis in human cancers through a previously unexpected mechanism.

Conflict of interest statement

None declared.

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References

1. Bornman PC, Beckingham IJ. Pancreatic tumours. *Br Med J* 2001, **322**, 721–723.
2. Rosewicz S, Wiedenmann B. Pancreatic carcinoma. *Lancet* 1997, **349**, 485–489.
3. Goggins M, Kern SE, Offerhaus JA, *et al*. Progress in cancer genetics: lessons from pancreatic cancer. *Ann Oncol* 1999, **10**(Suppl. 4), 4–8.
4. Barton CM, Staddon SL, Hughes CM, *et al*. Abnormalities of the p53 tumour suppressor gene in human pancreatic cancer. *Br J Cancer* 1991, **64**, 1076–1082.
5. Bramhall SR. The use of molecular technology in the differentiation of pancreatic cancer and chronic pancreatitis. *Int J Pancreatol* 1998, **23**, 83–100.
6. Tada M, Omata M, Ohto M. Clinical application of ras gene mutation for diagnosis of pancreatic adenocarcinoma. *Gastroenterology* 1991, **100**, 233–238.
7. Yanagisawa A, Ohtake K, Ohashi K, *et al*. Frequent c-Ki-ras oncogene activation in mucous cell hyperplasias of pancreas suffering from chronic inflammation. *Cancer Res* 1993, **53**, 953–956.
8. Hiyama E, Kodama T, Shinbara K, *et al*. Telomerase activity is detected in pancreatic cancer but not in benign tumours. *Cancer Res* 1997, **57**, 326–331.
9. Uehara H, Nakaizumi A, Tatsuta M, *et al*. Diagnosis of pancreatic cancer by detecting telomerase activity in pancreatic

juice: comparison with K-ras mutations. *Am J Gastroenterol* 1999, **94**, 2513–2518.
10. Aaronson SA. Growth factors and cancer. *Science* 1991, **254**, 1146–1153.
11. Pear WS, Nolan GP, Scott ML, *et al*. Production of high-titer helper-free retroviruses by transient transfection. *Proc Natl Acad Sci USA* 1993, **90**, 8392–8396.
12. Terada T, Ohta T, Sasaki M, *et al*. Expression of MUC apomucins in normal pancreas and pancreatic tumours. *J Pathol* 1996, **180**, 160–165.
13. Yoshida K, Ueno S, Iwao T, *et al*. Screening of genes specifically activated in the pancreatic juice ductal cells from the patients with pancreatic ductal carcinoma. *Cancer Sci* 2003, **94**, 263–270.
14. Onishi M, Kinoshita S, Morikawa Y, *et al*. Applications of retrovirus-mediated expression cloning. *Exp Hematol* 1996, **24**, 324–329.
15. Yoshizuka N, Moriuchi R, Mori T, *et al*. An alternative transcript derived from the trio locus encodes a guanosine nucleotide exchange factor with mouse cell-transforming potential. *J Biol Chem* 2004, **279**, 43998–44004.
16. Wu X, Noh SJ, Zhou G, *et al*. Selective activation of MEK1 but not MEK2 by A-Raf from epidermal growth factor-stimulated Hela cells. *J Biol Chem* 1996, **271**, 3265–3271.
17. Bonner TI, Kerby SB, Sutrave P, *et al*. Structure and biological activity of human homologs of the raf/mil oncogene. *Mol Cell Biol* 1985, **5**, 1400–1407.
18. Ishikawa F, Sakai R, Ochiai M, *et al*. Identification of a transforming activity suppressing sequence in the c-raf oncogene. *Oncogene* 1988, **3**, 653–658.
19. Hoyle PE, Moye PW, Steelman LS, *et al*. Differential abilities of the Raf family of protein kinases to abrogate cytokine dependency and prevent apoptosis in murine hematopoietic cells by a MEK1-dependent mechanism. *Leukemia* 2000, **14**, 642–656.
20. Davies H, Bignell GR, Cox C, *et al*. Mutations of the BRAF gene in human cancer. *Nature* 2002, **417**, 949–954.
21. Lee JW, Soung YH, Kim SY, *et al*. Mutational analysis of the ARAF gene in human cancers. *APMIS* 2005, **113**, 54–57.
22. Beck TW, Huleihel M, Gunnell M, *et al*. The complete coding sequence of the human A-raf-1 oncogene and transforming activity of a human A-raf carrying retrovirus. *Nucleic Acids Res* 1987, **15**, 595–609.