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Sensitive Ewing sarcoma and neuroblastoma cell lines have increased levels of BAD expression and decreased levels of BAR expression compared to resistant cell lines

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Abstract

The purpose of the study is to compare the mRNA expression of pro-apoptotic and anti-apoptotic genes by using the GeneSystem 320 (Capital Genomix Inc., Gaithersburg, MD) to examine the differential expression in Ewing sarcoma and neuroblastoma cell lines. This is an alternate method for which internal controls have been built into the system for comparing mRNA. The tumor cell lines were chosen based on their previously characterized Fas-resistance or Fas-sensitive properties in order to determine the differences in their response to apoptotic signals. Two representative pro-apoptotic genes (BAD and SMAC) and one anti-apoptotic gene (BAR) were chosen for the study. The results of mRNA expression were correlated with protein expression by Western analysis. BAD was highly expressed in the Fas-sensitive cell lines while SMAC was equally expressed in both Fas-sensitive and Fas-resistant cell lines. On the other hand, BAR was highly expressed in Fas-resistant cell lines and minimally expressed in the Fas-sensitive cell lines. Our data suggests that levels of BAD and BAR mRNA expression predict sensitivity to apoptosis.

Keywords: Apoptosis; Neuroblastoma; Ewing sarcoma; GS320 system; RAGEtags; BAR; BAD; SMAC

1. Introduction

Differences in gene expression levels have been associated with clinical parameters, response to chemotherapeutic agents and patient survival [1], and gene expression profiling studies have been conducted in various tumors and genetic diseases using cDNA microarray analysis. Thousands of genes can be analyzed simultaneously by cDNA microarray screening and powerful software and bioinformatics tools are available to interpret the results. However, it is relatively expensive and unnecessary to analyze thousands of genes when only a few of them are involved in a particular disease. A rapid, reliable and less costly technique may therefore, be more appropriate to assess the gene expression profile of clinical samples. In this study, we employed rapid analysis of gene expression (RAGE) technology using the GeneSystem 320 (Capital Genomix Inc., Gaithersburg, MD) to examine the differential expression of apoptosis-related genes in Ewing sarcoma and neuroblastoma cell lines.

The susceptibility of cells to apoptotic signals depends on the ratio between the anti-apoptotic and the multi-domain pro-apoptotic members [2]. The overall ratio of the pro-apoptotic to anti-apoptotic

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BCL-2 members determines the susceptibility of cells to death signals from either TNF/Fas ligands or drugs [3].

We compared the expression of pro-apoptotic proteins, such as BAD, and SMAC and anti-apoptotic protein, such as BAR in Ewing sarcoma and neuroblastoma cell lines with known sensitivity or resistance to see if overexpression or downregulation of these genes correlates with Fas/TRAIL-sensitivity or resistance of these tumor cells.

BAD is a pro-apoptotic member of the Bcl-2 family of proteins that exerts a death-promoting effect by heterodimerization with Bcl-X(L), nullifying its anti-apoptotic activity [4].

SMAC (second mitochondria-derived activator of caspase) deregulates the inhibitor of apoptosis proteins and contributes to the death of a cell if the downstream apoptotic pathway is blocked [5].

BAR (bifunctional apoptosis regulator), a membrane bound protein, suppresses BAX-induced apoptosis [6] in yeast and mammalian cells. In addition, it binds caspases and suppresses Fas-induced apoptosis. It is therefore, conceivable that resistant tumor cells may have high levels of BAR expression.

2. Methods

2.1. Cell lines

The Ewing sarcoma cell lines used in the study were SKNMC, 5838, CHP100L (Fas-sensitive), TC248, TC268, TC32, CHP100S, SKNMCR and A4573 (Fas-resistant) [7,8]. The neuroblastoma cell lines used were IMR32, TC378, CHP126 (Fas-resistant) [9] and LAN (TRAIL-resistant) [10].

To examine the expression of SMAC and BAD genes, A/B RAGEtag synthesis was performed for IMR-32, LAN, 5838, SKNMCR, SKNMC and TC248 cell lines. To examine BAR expression in cell lines, B/A RAGEtag synthesis was performed for IMR32, TC378, 5838, A4573, SKNMC and CHP126 cell lines.

Tumor cell lines were grown in RPMI with 10% fetal calf serum, 0.1% fungizone at 37 °C in a humidified atmosphere containing 5% CO_2 for several days in 75 ml flasks till they were confluent. They were harvested by adding trypsin for 5 min and the reaction was inactivated by adding 2 ml of fetal calf serum. The cells were washed with DMEM media without serum and pelleted in a clinical centrifuge for 2 min. The media was aspirated and cell pellets were stored at −80 °C until ready for RNA extraction.

2.2. RNA extraction

Cell pellets were extracted with 1 ml Trizol (GIBCO, Gaithersburg, MD) according to the manufacturer's protocol, then placed in a microtube and 0.2 ml chloroform was added. The mixture was then shaken for 15 s and incubated at room temperature (RT) for 3 min. Centrifugation was performed at 12,000×*g* for 15 min at 4 °C. The upper aqueous phase was transferred to a new tube and 0.5 ml propanol was added. The whole mixture was vortexed and incubated at RT for 10 min before centrifugation at 12,000×*g* for 10 min at 4 °C.

The supernatant was decanted and washed with 75% ethanol and vacuum dried using a SpeedVac for 10 min. The RNA pellet was then dissolved in 50 μl water (RNAse-free) by vigorous pipetting. The mixture was incubated at 55–60 °C for 10 min. RNA was then stored at −80 °C.

2.3. cDNA synthesis

At least 10 μg of RNA from each sample was used in the synthesis of full-length cDNA. The RNA was pre-treated with DNA-free™ (Ambion, Inc., Austin, TX) to remove any DNA. To synthesize the first strand cDNA, 6.5 μl of biotinylated oligo(dT) (GS320 RAGEtag Synthesis Kit, Capital Genomix, Inc., Gaithersburg, MD) was used as the primer for each reaction. We used a cDNA synthesis kit Superscript Choice System from Life Technologies (Gaithersburg, MD) to synthesize the double stranded cDNA according to the manufacturer's instruction. In order to remove any excess RNA, 5 μl of RNAse A/T1 (Ambion, Inc., Austin, TX) was used for each reaction mixture. After phenol/chloroform extraction and ethanol precipitation, the cDNA pellet was redissolved with 10 μl TE buffer.

2.4. cDNA quality test

Aliquots of 5 ng of each cDNA were then tested for full-length synthesis with a cDNA Integrity Kit (KPL Inc., Gaithersburg, MD) according to the manufacturer's direction. Briefly, primer sets of house keeping genes included in the kit were used for PCR, e.g. Clathrin 3′, Clathrin 5′, S6, L3, GAPDH 3′ and GAPDH 5′. After the PCR, 5 μl of PCR products were loaded onto 10% TBE polyacrylamide gel and electrophoresed. The gel was then stained with SYBR Green 1 (1:10,000) (Molecular Probes, Eugene, OR) and the bands were visualized with a STORM Imager (Molecular Dynamics, Sunnyvale, CA). PCR product sizes for Clathrin 3′ (550 bp), Clathrin 5′ (570 bp), S6 (600 bp), L3 (468 bp), GAPDH 5′ (887 bp) and GAPDH 3′ (540 bp) were obtained for each sample. The quality and integrity of cDNA produced had to be tested before syntheses of RAGEtags can be carried out. The cDNAs for each sample were tested with the kit and shown to be full length according to manufacturer's directions. Once the integrity of the cDNA was checked, we proceeded to synthesize RAGEtags with the GS320 System (Capital Genomix, Inc., Gaithersburg, MD).