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A cancer/testis antigen, NY-SAR-35, induces EpCAM, CD44, and CD133, and activates ERK in HEK293 cells

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ABSTRACT

The cancer/testis (CT) antigen NY-SAR-35 gene is located on the X chromosome and is aberrantly expressed in various cancers but not in normal tissues, other than testes. Previously, we reported the expression of NY-SAR-35 enhanced cell growth, proliferation, and invasion in HEK293 and cancer cells. To extend understanding of the NY-SAR-35 gene, we used a next generation sequencing (NGS) approach. NY-SAR-35 expression induced growth, proliferation, metastasis, and stemness genes, as indicated by the up-regulations of CXCR4, EpCAM, CD133, and CD44, at the mRNA and protein levels. The expression of NY-SAR-35 in HEK293 cells significantly increased ERK phosphorylation, but not the phosphorylation of AKT. In HEK293/NY-SAR-35 cells, the expressions of pro-apoptotic proteins, including p53, Bax, and p21, were reduced and that of cyclin E was increased. Also, NY-SAR-35 increased the expressions of pluripotency genes (Nanog, Oct-4, and Sox2) and the ability of HEK293 cells to form colonies. Taken together, the present study indicates NY-SAR-35 functions as a CT antigen that triggers oncogenesis and self-renewal.

1. Introduction

Cancer/testis (CT) antigens are a heterogeneous group of more than 200 proteins with an eponymous expression pattern restricted to tumor cells of different histological origin and to germ cells in testis and placenta [1–4]. CT antigens are classified according to whether they are encoded on the X chromosome (the CT-X genes) or not (the non-X CT genes) [5]. Many CT antigens have been characterized, but their functions during gametogenesis and carcinogenesis remain poorly understood [2,4]. Recent studies have indicated CT antigens, including CT45A [6] and SSX [7], are probably involved in cell cycle regulation, cell survival, apoptosis, and metastasis [2,8,9], and this new insight has the potential to result in the developments of more effective cancer vaccines [10].

One of the CT antigens, NY-SAR-35 (also called Fragile X Mental Retardation 1 Neighbor or FMR1NB) was identified using a serological analysis of recombinant cDNA expression (SEREX) based method [11]. NY-SAR-35 is a 255 amino acid multi-pass membrane protein with a predicted molecular mass of 29.2 kDa, and is encoded by a gene located on the human X chromosome [11]. NY-SAR-35 has been found to be aberrantly expressed in various cancers, including melanoma, sarcoma, lung cancer, breast cancer, and ovarian cancer [11]. Furthermore, an analysis of the methylation status of the NY-SAR-35 gene showed that its expression might be regulated by methylation of its promoter region [12]. Recently, we reported NY-SAR-35 expression enhanced the cell growth, proliferation, and invasiveness of HEK293, SNU-449 (a hepatocellular carcinoma cell line) and SK-LC-14 (a lung cancer cell line) cells [13,14].

In the current study, to extend our understanding of the NY-SAR-35 gene, we used next generation sequencing (NGS) to analyze the differential gene expression transcriptomes of HEK293 and HEK293/NY-SAR-35 cells.

2. Materials and methods

2.1. Cell culture

The human embryonic kidney (HEK) 293 cells were obtained from the American Type Culture Collection (ATCC). HEK 293 cells

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exogenously expressing NY-SAR-35 (HEK293/NY-SAR-35 cells) were obtained as previously described [13]. Cell lines were maintained and cultured in DMEM (Sigma Aldrich, St. Louis, MO, USA) supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were cultured in a humidified 5% CO_2 atmosphere at 37 °C.

2.2. RNA isolation and RT-PCR

Total RNA was extracted from cells using the RNeasy mini kit (Qiagen, Hilden, Germany). Total RNA of cDNA was synthesized from 1 μg total RNA using M-MLV reverse transcriptase (Promega, WC, USA). PCR products obtained by PCR amplification were analyzed by agarose gel electrophoresis and visualized using ethidium bromide. All primer sequences and conditions used for PCR are listed in Supplementary Table 1.

2.3. Western blot analysis

Cells were lysed in lysis buffer and cellular debris was removed by centrifugation at 15,000 rpm for 10 min. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes (Hybond-ECL, GE Healthcare, USA), blocked with 5% skim milk, and blotted with appropriate primary antibodies (Supplementary Table 2). Membranes were then incubated with horseradish peroxidase-conjugated goat anti-rabbit-IgG or goat anti-mouse-IgG secondary antibodies for 1 h at room temperature and detected by enhanced chemiluminescence (PerkinElmer Life science, MD, USA).

2.4. Soft agar colony formation

To examine anchorage independent growth, cells were suspended at a density of 500 cells per well in medium containing 0.35% agar overlaid into 96-well plates with a 0.5% agar base. All samples were plated in triplicate. Colonies of >100 μm in diameter were counted and imaged using a microscope (Olympus).

2.5. Flow cytometry analysis and sorting

The expression profiles of EpCAM, CD133, and CD44 in cultured cells were analyzed by flow cytometry. Cells were stained with a solution containing PerCP-Cy™ 5.5-conjugated anti-EpCAM (BD Bioscience), PE-conjugated anti-CD133 (Miltenyi Biotech), and FITC-conjugated anti-CD44 (BD Pharmingen). Corresponding isotype-matched mouse immunoglobulins were used as negative controls (BD Bioscience). Labeled cells ($\geq 1 \times 10^4$ cells) were resuspended in PBS, and analyzed by FACS Canto II (BD Bioscience). Using the same setup, CD133 and CD44 co-labeled HEK293/NY-SAR-35 cells were sorted by FACS Aria (BD Bioscience) to obtain CD133+CD44+, CD133+CD44-, CD133-CD44+, and CD133-CD44- subpopulations.

2.6. Next generation sequencing (NGS)

Total RNAs were extracted from cultured HEK293 and HEK293/NY-SAR-35 cells and sequenced at a commercial sequencing facility (Macrogen, Seoul, Korea). TopHat aligns RNA sequences to mammalian-sized genomes using the ultra high-throughput short read aligner Bowtie and Cufflinks was then used to estimate the relative abundances of the transcripts. The differential gene expressions of these two RNA samples were analyzed by transcriptome sequencing (total 37,357 human genes).

2.7. Statistical analysis

Values are presented as the means ± standard deviations of three independent experiments. Differences were analyzed using the Student's t-test. The analysis was performed using the SPSS statistical package (version 14.0; SPSS Inc., IL, USA). P values of <0.05 were considered to indicate statistically significant differences.

3. Results

3.1. Effects of NY-SAR-35 on gene expression pattern in HEK293 cells

Previously we reported the expression of NY-SAR-35 promoted the cell proliferation, migration, and invasion of HEK293 cells [13]. To identify more phenotypic effects induced by NY-SAR-35 overexpression, NGS was used to identify genes differentially expressed in the transcriptomes of HEK293 and HEK293/NY-SAR-35 cells. Using a ≥2-fold change as a cutoff, transcriptome sequencing indicated that 866 of 37,357 genes were upregulated and 736 genes were downregulated in HEK293/NY-SAR-35 cells. Several genes related to stemness, cell proliferation, migration, and metastasis were upregulated in HEK293/NY-SAR-35 cells (Table 1). We focused on these the upregulated genes in the present study and confirmed their up-regulations by RT-PCR (Supplementary Fig. 1). We found NY-SAR-35 overexpression upregulated CXCR4 (a metastatic gene) [15], but not those of other metastatic genes, including TWIST1 and CDH2, (Supplementary Fig. 1). Additionally, Akt1 and CLSTN1 (both cell proliferation-related genes) were upregulated, and EpCAM, CD44, and CD133, which are responsible for proliferation, metastasis, and stemness, were significantly up-regulated as compared with control HEK 293 cells (Fig. 1A and D).

3.2. NY-SAR-35 increased EpCAM, CD44, and CD13 protein levels

Western blotting and flow cytometry assays showed EpCAM expression was significantly higher in HEK293/NY-SAR-35 cells than in HEK293 cells (Fig. 1B and C). More specifically, NY-SAR-35 overexpression strongly increased levels of EpCAM and of its glycosylated isoform (35 and 39 kDa, respectively) (Fig. 1B). Flow cytometry showed HEK293/NY-SAR-35 cells increased EpCAM protein levels by 97.9 ± 2.6% as compared with HEK293 controls

Table 1
General functions of representative genes up-regulated in HEK293/NY-SAR-35 cells. NY-SAR-35 overexpressing HEK293 and HEK293 cells were subjected to transcriptome analysis by next-generation sequencing to investigate gene expression patterns. Genes up-regulated by ≥ 2 fold were analyzed.

Functions	Gene Names				
Transcription regulation	HKR1	MAZ	WAC	HNRNPD	
Growth	BIRC5	SIRT2	DDX3X	OCIAD2	DDX17
	DHX32	Akt1	Akt2	CD133	
Proliferation	Akt1	Akt2	CLSTN1	RBBP7	SMARCA4
	CXCR4	MAPK1	NPM1	EpCAM	KIDINS220
	CD44	CD133			
Differentiation	RBBP7	FBXO7	MAPK1	TWIST1	KIDINS220
	EpCAM	CXCR4	DSE	CDH2	
Apoptosis	CLK2	SLFN11	CXCR4		
Adhesion	EpCAM	LSR	LPHN2	CDH2	CD44
Migration	CLSTN1	CD44	CD133	TWIST1	ARHGEF7
	CDH2	CXCR4			
Immunoregulation	FKBP4				
Oncogenesis	EpCAM	RAB34	DDX3X	RAB4B	
Repair mechanism	HNRNPK	UBE2D3			
Unknown	EXOSC6				

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