Promising markers for the detection of premature senescence tumor cells induced by ionizing radiation: Cathepsin D and eukaryotic translation elongation factor 1

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1. Introduction

Recently, it has been proved that induction of senescence could be a promising way of tumor treatment. Senescence was originally described in normal human cells undergoing a finite number of divisions before permanent growth arrest [1, 2]. It has now become regarded more broadly as a general biological program of terminal growth arrest. A variety of stresses such as ionizing radiation (IR), oxidative stress, oncogenic transformation, DNA damaging agents triggers stress-induced premature senescence, i.e. rapid and permanent cell growth arrest. Therefore, premature senescence is bona fide barrier to tumorigenesis and hallmark of premalignant tumors. However, there is lack of obvious markers for senescent tumor cells. To identify useful premature senescence markers for tumor cells, we monitored the changes of protein expression profile in IR-induced premature senescence MCF7 human breast cancer cells. We identified biomarkers which evidently changed their expression levels in ionizing radiation-induced senescenct tumor cells.

2. Methods and Results

2.1 Preparation of protein lysates

Protein lysates were prepared in Lysis buffer (9M Urea, 2M thio Urea, 100mM DTT, 2% CHAPS (w/v), 60 mM *n*-octyl-D-glucopyranoside, 2% IPG buffer (pH 3-10, Amersham biosciences, Piscataway, NJ) containing protease inhibitor). After centrifugation at 14,000 rpm, 10min, 4 °C, the supernatant was taken for the subsequent 2-dimensional gel electrophoresis.

2.2 Two dimensional electrophoresis

The isoelectric focusing of each sample containing equal amount of protein (120 μg) was conducted on a linear wide-range immobilized pH gradient (pH 3-10) with a total focusing time of 81,780Vhr, at 20°C, using the IPGphor system in accordance with the manufacturer's instructions (Amersham Biosciences, Piscataway, NJ). The second dimension was then conducted on SDS-PAGE gels (11% polyacrylamide, 0.26% 1,4-Bis (acryloyl) piperazine (PDA) / 25.5 cm × 19.6 cm × 1 mm), under constant current, in three steps (Step 1: 5 w/gel; Step2: 10 w/gel; step 3: 15 w/gel) at 20°C, using an Ettandalt 6 system (Amersham Biosciences). The analytical gels were stained using the PlusOne^{TM} silver staining kit (Amersham Biosciences) in accordance with the manufacturer's instructions, with the exception of the glutaraldehyde treatment [3].

2.3 In-gel protein digestion and electrospray ionization tandem mass spectrometry (ESI-MS/MS)

Proteins were subjected to in-gel trypsin digestion. The excised gel spots were then destained and incubated using 200 mM ammonium bicarbonate for 20 min. These gel pieces were dehydrated and dried, and then rehydrated. The peptide solution was desalted with a C_{18} nano column (IN2GEN Co., Ltd.).

The MS/MS of peptides generated via in-gel digestion was conducted by nano-ESI on a Q-TOF2 mass spectrometer (Micromass, Manchester, UK). The product ions were analyzed with an orthogonal TOF analyzer. The data were processed with a Mass Lynx Windows NT PC system.

In order to identify the proteins, all MS/MS spectra recorded on tryptic peptides derived from the spots were searched against protein sequences from the NCBInr databases, using the MASCOT search program (www.matrixscience.com).

2.4 Western Blotting and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

For Western Blot assay, Cells were collected in extraction buffer (25 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1% Triton X-100, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 mM sodium azide, 1 mM orthovanadate, 20 μg/ml aprotinin, and 3 mM dithiothreitol). Equal amount of proteins was loaded onto an 8 or 10% SDS-PAGE gel. Proteins in gels were transferred to a nitrocellulose membrane. The membrane blot was incubated with specific primary antibodies and secondary antibodies, and an enhanced chemiluminescence substrate kit (Amersham Bioscience, NJ).

For RT-PCR, total RNA was prepared using TRIzol reagent (Invitrogen). Total RNA was used as template for cDNA synthesis with M-MLV RT using SuperScript^{TM} III reverse transcrptase kits (Invitrogen) Subsequently, PCR cycles were performed by 28 cycles.

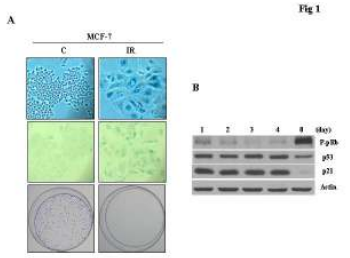

Figure 1. (A) Morphological changes of IR-induced premature senescence cells. (B) Western blot analysis of cell cycle regulatory genes in human breast cancer MCF7 cells.

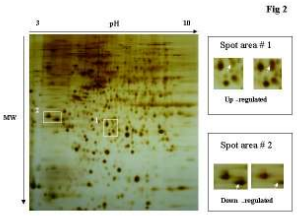

Figure 2. Two-dimensional gel electrophoresis image of proteins extracted from IR-induced premature senescence MCF7 cells.

Table 1. Identification of differentially expressed protein spots in IR-induced senescence MCF7 cells by LC-MS/MS

Spot No.	Protein	NCBI access no.	Mass	Mascot score
1	Eukaryotic translation elongation factor 1 beta 2	BC067787	24919	98
2	Chain B, Cathepsin D	BC016320	26457	60

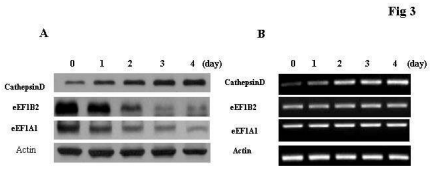

Figure 3. (A) Changes of protein levels in IR-induced premature senescence MCF7 cells. (B) Reverse Transcriptase-Polymerase Chain Reaction with specific probes for cathepsin D, eEF1A1, and eEF1B2 in MCF7 cells.

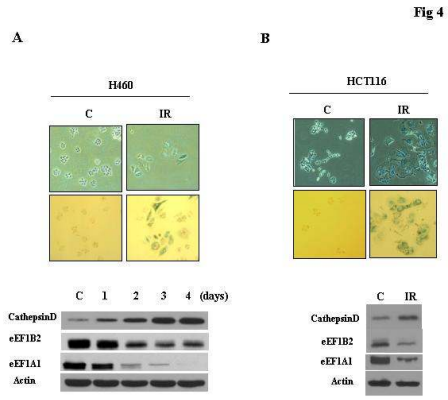

Figure 4. (A) Morphological changes and protein levels in IR-induced premature senescence H460 cells. (B) Morphological changes and protein levels in IR-induced premature senescence HCT116 cells.

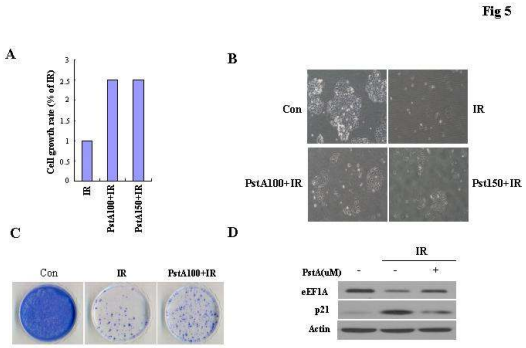

Figure 5. Effects of pepstatin A, cathepsin inhibitor on cell growth rate (A), proliferation (B), colony formation (C), eEF1A1 and p21 expressions (D).

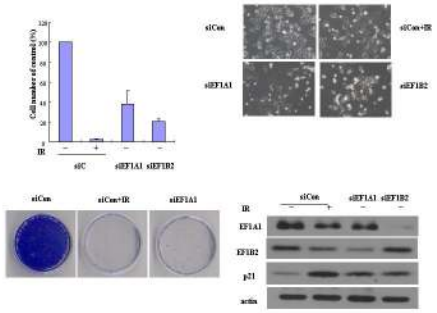

Figure 6. Effects of RNA interference of eEF1A1and eEF1B2 on cell growth rate (A), proliferation (B), colony formation (C), and p21 expressions (D).

3. Conclusion

We conducted comparative proteomic analysis to identify differentially expressed proteins in IR-induced senescence MCF7 cells. Cathepsin D was up-regulated in the transcriptional level, and eEF1B2 and eEF1A1 were down-regulated in the posttranslational levels during the process of IR-induced premature senescence pathway. Such altered expressions were confirmed in three different cancer cell types (human colon cancer HCT116, breast cancer MCF7, and non-small cell lung cancer H460 cells). Cathepsin D and eEF1A1 has been evidenced critical roles in IR-induced premature senescence pathway either with inhibitor or with RNA interference assay. Therefore, we conclued that these proteins will be useful as prognostic biomarkers .for the radiotherapy.

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