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Expression of carbonic anhydrase IX & XII in ovarian cancer tissues

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ABSTRACT

Ovarian cancer is a potentially life-threatening malignancy that develops either in one or both the ovaries. Ovarian cancer is considered as a "silent killer." Among many tumor markers which have been identified in many tumors, Carbonic anhydrase IX & XII are unique member of the CA family and suggested to play a vital role in oncogenic processes. CA IX participates in the regulation of acid-base balance, cell proliferation, adhesion and malignant processes. In this study mRNA transcription was studied by reverse transcriptase PCR (RT-PCR).The products of PCR were run on agarose gel electrophoresis which showed strong positive expression of CA IX & XII. The expression pattern of CA IX & XII suggested that they could also serve as a histopathological marker protein for hypoxia in malignant ovarian tumors.

KEYWORDS

Carbonic anhydrase;
Hypoxia;
Ovarian cancer;
Tumor markers;
CA-125.

INTRODUCTION

Ovarian Cancer is the fifth leading cause of cancer death in women, the leading cause of death from gynecological malignancy, and the second most commonly diagnosed gynecologic malignancy. Ovarian cancer causes damage in tissues of the ovary (one of a pair of female reproductive glands in which the ova or eggs, are formed). Most ovarian cancers are either ovarian epithelial carcinomas (cancer that begin in the cells on the surface of the ovary) or malignant germ cell tumors (cancer that begin in egg cells).

Ovarian cancer accounts for about 3% of all cancers in women. The American Cancer Society estimates that about 21,550 new cases of ovarian cancer will be diagnosed in the United States during 2009.

MARKERS FOR OVARIAN CANCER

The high overall mortality from ovarian cancer is primarily due to delay in diagnosis-although 90% of patients with early stage disease can be cured, 70% of patients at diagnosis have stage III or higher disease, where the 5 years survival rate is only 20%. At present, there is a dreath of clinically useful antigenic markers for early stage diseases.

The blood test called CA-125 is used in differential diagnosis and in follow up of the disease, but it has not been shown to be an effective method to screen the early-stage ovarian cancer and is currently not recommended for this use.

Researcher's eye focused on Carbonic anhydrase, which turned to be a best tumor marker in cancer tis-

sues. The carbonic anhydrases (CAs) comprise a family of evolutionarily ancient enzymes found ubiquitously in natures. They have important roles in facilitating transport of carbon dioxide and protons in the intracellular space, across biological membranes and in the unstirred layers of the extra cellular space[1]. At least 15 different alpha-carbonic anhydrase isoforms were isolated in mammals, where these zinc enzymes play crucial physiological roles. Some of these isozymes are cytosolic (CA I, CA II, CAIII, CAVII, CAXIII) others are membrane bound (CA IV-CA IX, CA XII, CA XIV and CA XV), CA VA and CA CB are mitochondrial and CA VI is secreted in saliva and milk[2].

Carbonic anhydrases (CAs) are zinc-containing metalloenzymes whose main function is to participate in the regulation of acid-base balance. Mammals have at least 12 active isozymes that belong to α-CA family. The CA isozymes differ in their subcellular localization, kinetic properties, and inhibition profiles. In addition, each CA isozyme has a unique distribution in tissues[3]

The carbonic anhydrases (CAs) catalyze the reversible hydration of carbon dioxide $CO_2 + H_20 ? HCO_3 +$ H+. At present 13 isozymes, differ in their tissue distribution and enzymatic activity. CA influence intra and extra cellular pH and ion transport in varied biological processes. It is recently identified CA IX and CAXII as hypoxia inducible gene[4]

CA are involved in bone resorption, gluconeogenesis, ureagenesis and the formation of gastric acid, saliva and cerebrospinal fluid and have been suggested to play a significant role in epithelial cell interaction and cell proliferation[5]

CA IX was first recognized as the novel tumor – associated antigen, MN, in several human carcinoma and in normal gastric mucosa[6]. CA IX has been considered as a potential biomarker for tumor progression[7]. CA IX, a transmembrane isoform with predominant association with tumors and limited distribution in normal tissues, is strongly over expressed by hypoxia[8]

CA IX has been proposed to play a role in malignant processes since many tumors over express this enzyme. Because of their rapid growth, tumors commonly experience hypoxia (limited oxygen supply) since they initially have no extensive capillary network to supply the tumors cells with oxygen. As a result, cancer cells more than 100-200μm from nearest capillaries depend on anaerobic glycolysis for much of their energy production[9]

CA IX is upregulated by hypoxia and the study suggested that it may be one of the key molecules in tumor invasiveness and metastasis[10]. CA IX has been detected in the human cervical carcinoma cell line HeLa and also in carcinomas of ovary, endometrial and uterine cervix, but not in normal tissues from corresponding organs or from placenta.[11]

Recent progress in understanding the role of catalytically active carbonic anhydrase IX tumors has opened new possibilities for diagnostic and/ or therapeutic applications on carbonic anhydrase inhibitors selectively blocking the enzyme activity of cancer related isoforms[12]

The high expression of CA XII in some selected cancer cell lines suggested that it could serve as a useful biomarker of some malignant tumors and could also be considered as potential target for novel therapeutic applications[13] CA XII is a transmembrane isozyme that has been identified in carcinoma.[14]

EXPERIMENTAL

Sample collection

The samples were collected from Coimbatore Medical College Hospital, and G.Kuppusamy Naidu Memorial Hospital, Coimbatore. It has been approved by Ethical Committee. (GKNMH/IEC/2008 dt.15.11.2008). The samples were collected fresh in saline and stored at -80° C.

Isolation of RNA

Total RNA was isolated from different ovarian cancer tissues using the Trizol reagent (GIBCO-BRL) according to the manufacturers' instructions. To evaluate the suitability of purified RNA (human) for RT-PCR applications a control RT-PCR can be performed using template RNA and the provided control GAPDH primers. The GAPDH-specific control PCR primers are designed to be complementary to human GAPDH genes and generate a 496bp RT-PCR product.

Preparation of first strand cDNA

To 5 μl of RNA, 1 μl of oligo $(dT)_{18\ primer}$ (0.5 mg / ml)was added and 12 μl of nuclease-free de-ionized

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water was added and mixed gently for 3-5 seconds in a micro centrifuge. The mixture was incubated at 70° C for 5 minutes, chilled on ice and the drops are collected by brief centrifugation. The tubes were placed on ice and the following reagents were added :4µl of 5x reaction buffer,0.5µl Ribonuclease inhibitor (40 µl /µl), 2µl of 10mM dNTP mix. The mixture was gently mixed and the drops were collected by brief centrifugation. The mixture is incubated at 42° C for 5 minutes. 2µl AmV reverse transcriptase (10 µ/µl) was added and the final volume is made to 20 µl. The mixture was incubated at 42°C for 60 minutes. The reaction was stopped by heating at 70°C for 10 minutes and Chilled on ice. The first strand cDNA synthesized can be used directly for PCR. The synthesized cDNA should be stored at -20° C.

Primers for cDNA synthesis

Synthesis of first strand cDNA can be primed with either Oligo(dt)18 primer, random primers or gene specific primers. Oligo(dt)18 primers cDNA synthesis from the poly(A) tail present at the 3'-end of eukaryotic mRNA.

Random Primers initiate cDNA synthesis from the total RNA population (rRNA and mRNA). Therefore, using random primers for first strand synthesis resulted in a greater complexity of the generated cDNA compared with the oligo(dt)18 primer. As a consequence, the sensitivity and specificity of subsequent PCR reactions may be reduced. There are several applications where it is beneficial to use random primers, such as cDNA synthesis using mRNAs without a poly(A) tail, or cDNA synthesis using poly(A)-enriched RNA samples.

Gene-specific primers were used to synthesize specific cDNA from a pool of total RNA or mRNA and it can be obtained.

PCR

The primers for the PCR reaction were designed by using the published information on CA IX mRNA, and purchased from Helini Biomolecules, Chennai. The forward primer (F1) was 5'-CAC CGT TTC CCT GCC GAG AT -3' and the reverse primer (R1) 5'-AGC TGT AGC CGA GAG TCA CC -3'. In CA XII of experiments, the forward primer (F2) was 5'-GGA CAG CAC TTC GCC GCC GA-3' and the reverse primer (R2) 5'-GTA GCG GTA ATA TTC AGC GG-3'. With these primers the PCR amplification product was predicted. To confirm the results and all the other reagents for the PCR reaction was added except the dNTP mix. 5µl of cDNA was used as template. The PCR denaturation step for 1 min followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 1 min 30 sec, was followed by a final extension at 72°C for 3 min. The control PCR reaction was performed with the following primers for ß-actin: the forward primer was 5'-GAA GAG CTA CGA GCT GCC-3' and the reverse primer was 5'-TGA TCC ACA TCT GCT GGA -3'. The control reaction was carried out according to the following protocol: 94°C denaturation step for 1 min was followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72 °C for 40 sec, was followed by final extension at 72°C for 3 min. The results of the PCR reaction were analyzed using a 1.2% agarose gel containing 0.1 µg/ml ethidium bromide with DNA standard (100-bp DNA Ladder; Medox biotech,Chennai,India)

PCR amplification of first strand cDNA

To 25µl of 2 X PCR master mixes, 2µl of cDNA from first strand reaction was added and 1µl of forward primer and reverse primer was also added and made up to 50µl with water. The PCR cycle followed by Initial denaturation at 94°C for2 min, then Denaturation at 94°C for 30sec, and for Primer annealing at 54°C for 30sec,followed by Primer extension at 72°C for 1.5min.And place the PCR tubes at 4°C 1min.

RESULTS & DISCUSSION

Expression of RNA

Researcher's examined the expression of RNA which was isolated from Ovarian cancer patients using Trizol reagent. Quantitative estimation and purity analysis of RNA was determined by using UV spectrophotometer. The isolated RNA was qualitatively estimated using agarose gel electrophoresis. (Figure 1) The Figure showed the expression of RNA in all six lanes. RNA which was isolated from different ovarian cancer tissues were subjected to reverse transcription as de-

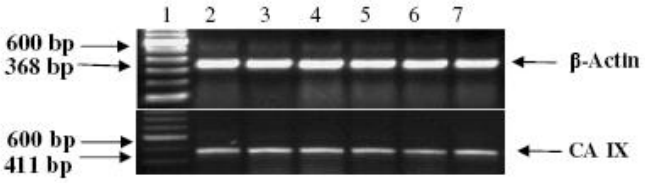
scribed in 'Methods'.

Lane 1 : Sample 1
Lane 2 : Sample 2
Lane 3 : Sample 3
Lane 4 : Sample 4
Lane 5 : Sample 5
Lane 76: Sample 6

Figure 1 : RNA Isolation from ovarian cancer tissues

Expression of CAIX gene in PCR products

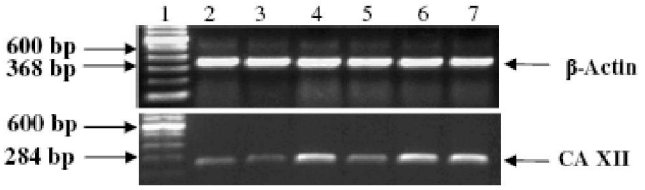
The PCR reaction products were separated on 0.8 % agarose gels and visualized by ethidium bromide staining. Stained gels were visualized under UV transilluminator.. The results showed the CA IX gene expression at the expected molecular weight in all the lanes when compared with the house keeping genes β actin.(Figure 2) The same was observed in CA XII gene expression (Figure 3)



Lane 1 : 100 bp DNA Ladder
Lane 2 : Sample 1
Lane 3 : Sample 2
Lane 4 : Sample 3
Lane 5 : Sample 4
Lane 6 : Sample 5
Lane 7 : Sample 6

Figure 2 : Expression of CA IX in ovarian cancer tissues

Since ovarian cancer produce clinical symptoms in its late stages, a clear need exists for better early detection methods. Although a number of tumor biomarkers for ovarian cancer have been identified and studied.[15] CA IX is present in several types of human tumors, whereas it is usually absent in the normal tissues from



Lane 1 : 100 bp DNA Ladder
Lane 2 : Sample 1
Lane 3 : Sample 2
Lane 4 : Sample 3
Lane 5 : Sample 4
Lane 6 : Sample 5
Lane 7 : Sample 6

Figure 3 : Expression of CA XII in ovarian cancer tissues

which these tumors originate.[16] It is possible that CA IX could represent a valuable diagnostic marker for ovarian mucinous tumors, as it is the case for pancreatic.[17] and colorectal[18] mucinous tumors, since they all show high levels of CA IX expression.

CA XII was originally discovered as a cell surface carbonic anhydrase isozyme that is over expressed in renal cell carcinoma.[19] In malignant tumors, CA XII has been proposed to acidify the immediate extracellular milieu surrounding the cancer cells. The acidification would create a microenvironment conducive tumor growth and spread.[20]

However, for selected cancers, some genes may proved to be a powerful diagnostic biomarkers. One such like is CA IX which was a biomarker for cervical dysplasia and carcinoma[21]. Previously it has been investigated that the expression of CA IX is an excellent diagnostic biomarker for renal cell carcinoma.[22] The combined detection of CA IX and CA XII expression will identify all renal cell carcinomas.[23] The results of our study showed the expression of CA IX & CAXII in all the ovarian cancer tissues which can be regarded as a best tumor marker in the early stages of ovarian cancer when compared with all other tumor markers. Finally, our studies suggest that CA XII and CA IX are highly expressed in ovarian tumors.

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