RESEARCH ARTICLE

SEMI QUANTITATIVE EXPRESSION ANALYSIS OF POTENTIAL CANCER BIOMARKERS

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ARTICLE INFO	ABSTRACT
	A cancer biomarker refers to a substance or process that is indicative of the presence of cancer it may be a molecule secreted by a tumor or a specific response of the body to the presence of cancer. **Background:** The aim of the present study is to investigate the feasibility and potential of the molecules Cytokeratin 20, Cytokeratin 17, Cytokeratin 10 and Anillin as tumor specific markers to rapidly detect cancer. **Materials and Methods**: The expression of biomarkers was analyzed by preparing RNA and synthesized cDNA. Specific primers were synthesized for the biomarkers Cytokeratin 10, Cytokeratin 20 Cytokeratin 17 and Anillin and semi quantitative gene expression was carried out using mMLuv reverse transcriptase. **Results**: All the three biomarkers showed an enhanced expression compared with normal fibroblasts while the expression of cytokeratin 10 did not show much expression in HCT116 while Cytokeratin 20 and Anillin showed better expression in both cell lines. **Conclusion**: All the biomarkers showed comparatively good expression indicating the use of these genes as potential makers for detection of cancer.

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INTRODUCTION

Cancer is a generic term for a large group of diseases that can affect any part of the body. Other terms used are malignant tumors and neoplasms. One defining feature of cancer is the rapid creation of abnormal cells that grow beyond their usual boundaries, and which can then invade adjoining parts of the body and spread to other organs. This process is referred to as metastasis. Carcinogenesis is a multistep process featuring the accumulation of several genetic alterations, including the activation of oncogenes and the inactivation of tumor-suppressor genes. The International Agency for Research on Cancer (IARC) estimates that there were some 12 million new cases in 2009 with more than 7 million deaths. WHO (World Health Organization) estimates that about one-third of the cancer burden could be decreased if cases were detected and treated early. Cancer cells display a broad spectrum of genetic alterations that include gene rearrangements, point mutations, and gene amplifications, leading to disturbances in molecular pathways regulating cell growth, survival, and metastasis.

When such changes manifest in majority of patients with a specific type of tumour, these can be used as biomarkers for detection and developing targeted therapies, besides predicting responses to various treatments (Anant Narayana Bhatt *et al.*, 2010). The National Cancer Institute defines a biomarker as "a biological molecule found in the blood, other body fluids, or tissues that is a sign of a normal or abnormal process or of a condition or disease. The ideal biomarker should screen for the disease and its progression, identify high-risk individuals, predict recurrence, and monitor response to treatments. It should be economical, consistent, non-invasive, easily accessible, and quickly quantifiable. (Vicki M.Velonas *et al.*, 2013)

Tumor Markers comprise a wide spectrum of biomacromolecules synthesized in excess concentration by a wide variety of neoplastic cells. The appearance of tumor marker and their concentration are related to the genesis and growth of malignant tumors in patients. An ideal tumor marker should be highly sensitive, specific, and reliable with high prognostic value, organ specificity and it should correlate with tumor stages. Many tumor markers have shown excellent clinical relevance in monitoring efficacy of different modes of therapies during entire course of illness in cancer patients.

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Compared to procedures such as radiology, cytology and endoscopy, the use of biomarkers as cancer screening tests have several advantages (Duffy *et al.*, 2001). Biomarkers can be measured in biological fluids such as blood and urine that can be obtained with minimal inconvenience to subjects undergoing screening. This in turn should lead to high compliance rates. Biomarkers are valuable drug development tools that provide more accurate or more complete information regarding disease progression. One of the most recently investigated markers is Cytokeratin 20 (CK 20), which was first characterized by Moll *et al.*, 1992. In tumor condition the CK20 level increases. CK-20 positivity was seen in a vast majority of adenocarcinomas of the colon, mucinous ovarian tumors, transitional-cell and Merkel-cell carcinomas and frequently also in adenocarcinoma of the stomach, bile system, and pancreas. Most squamous cell carcinomas in general and most adenocarcinomas from other sites (breast, lung, endometrium), nonmucinous tumors of the ovary, and small-cell lung carcinomas were essentially or completely negative (Roland Moll *et al.*, 1992). The expression of CK17 was restricted to pancreatic ductal carcinoma, the ampullary carcinoma of pancreatobiliary origin, and intrahepatic cholangiocarcinoma.Increased expression of keratins in endothelial cells, such as keratin 17, may contribute to the angiogenesis induced by HepG2 cells (Yong Xu *et al.*, 2004). The advantages of using Biomarkers are

- For many biomarkers, automated assays are available, thus allowing the processing of large numbers of samples in a relatively short period of time.
- Tests for biomarkers provide quantitative results with objective endpoints.
- Assays for biomarkers are relatively cheap.
- Early detection of cancer is based on the observation that treatment is more effective when cancer is detected earlier. Utilizing the potential of tumor markers in devising rapid and sensitive diagnostic tests is therefore of primary importance.

Keratins

Keratins are proteins ranging from 40KDa to 68 KDa are known to form intermediate filaments of 8-10 nm in diameter. Keratins are remarkably diverse, highly resistant and the most conserved cytoskeletal proteins present in all types of epithelial cells. The composition of keratin filaments ranges from a few polypeptides to 19 different polypeptides ranging from 40KDa to 68 KDa. There may be variations in keratin expression compared to that in normal tissue, depending on the degree of differentiation of epithelial tumors. This property of keratins allows their effective use, in combination with other changes, as tumor markers for malignant transformation in epithelioid tumors. Keratins as tumor markers have two main applications: (i) in distinguishing epithelial from nonepithelial tumors, and (ii) in distinguishing the type of epithelial tumor. The degree of keratin expression in tumors is remarkably high.

Cytokeratin 20

Cytokeratin 20 is 46kDa intermediate filament protein that has been identified with expression primarily restricted to gastric and intestinal epithelium, urothelium, and Merkel cells. Cytokeratin 20 is a unique type I keratin that is expressed in adenocarcinomas of the colon, stomach, pancreas and bile system. It is also expressed in mucinous ovarian tumors, transitional cell carcinomas of the urinary tract, and Merkel cell carcinomas (Amiram Fishman *et al.*, 2000). CK20 is essentially non-reactive in squamous cell carcinomas and adenocarcinomas of the breast, lung, and endometrium, as well as non-mucinous tumors of the ovary and small cell carcinomas.

Cytokeratin 17

Cytokeratin 17 a type I cytokeratin, is found in nail beds, hair follicles, sebaceous glands, and other epidermal appendages. CK17 was restricted to pancreatic ductal carcinoma, the ampullary carcinoma of pancreatobiliary origin, and intrahepatic cholangiocarcinoma.

Cytokeratin 10

Keratin, type I cytoskeletal 10 is a protein that in humans is encoded by the KRT1gene. Keratins are heteropolymeric structural proteins which form the intermediate filament. These filaments, along with actin microfilaments and microtubules, compose the cytoskeleton of epithelial cells. This gene encodes a member of the type I (acidic) cytokeratin family, which belongs to the superfamily of intermediate filament (IF) proteins.

Anillin

Anillin is an actin-binding protein that can bind septins and is a component of the cytokinetic ring. Anillin is expressed ubiquitously but with variable levels of expression, being highest in the central nervous system. Reports indicate that septins have a role in various disease states including cancer. The median level of anillin mRNA expression was not only shown to be higher in tumors compared to normal tissues but also increase as disease progressed. Anillin is expressed ubiquitously but with variable levels of expression, being highest in the central nervous system. The median level of anillin mRNA expression was higher in tumors than normal tissues except in the central nervous system where anillin mRNA levels were lower in tumors. (Peter Hall *et al.*, 2005). To establish alternative approaches with higher sensitivity, polymerase chain reaction (PCR)-based assays that have been used. PCR techniques are highly sensitive and clinically useful for detecting cancer markers in circulating tumor cells (Chie Suzuki *et al.*, 2005). The aim of the present study is to investigate the feasibility and potential of the molecules Cytokeratin 20, Cytokeratin 17, Cytokeratin 10 and Anillin as tumor specific markers to rapidly detect cancer by semi quantitative PCR. This will not only be of great significance in developing a reliable diagnostic method for screening multiple cancers, but also serve as valuable disease prognosis indicators and potential vaccine/ drug targets.

MATERIALS AND METHODS

The cell lines SW480 and HCT116, ATCC strains were provided by IISc, Bangalore. Afibroblast, fetal fibroblasts were established post biopsy in the Cell Culture Lab, ENZENE Biosciences pvt ltd All the cell lines were grown in monolayer in appropriate media DMEM (Hyclone) supplemented with

10% FCS (Gibco) andwere maintained at 37°C in an atmosphere of humidified airwith 5% CO$_2$. Trypsin EDTA (Gibco) was used to detach the monolayer for subculturing or for preparing cells for experiments.. After 72 hours total RNA was isolated by using the RNeasy mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions

- *SW480*- Human colorectal adenocarcinoma cells
- *HCT116*-Humancolorectal carcinoma cells
- Afibroblast- Human normal epithelial cells

These cells were maintained in DMEM-10% [10% FCS] +antibiotics. SW480, HCT116 are colorectal carcinoma cells, whereas Afibroblast is a normal epithelial cell. Approximately 1 million cells of each cell lines were suspended in 200µl RLT Buffer each for RNA extraction according to [QIAGEN RNA extraction kit].The RNA extracted was run on 1.2% Formaldehyde agarose gel.

Formaldehyde agarose gel electrophoresis

For gel preparation (1.2%) 0.24g Agarose was added to 20ml 1XFAbuffer, 360µl formaldehyde and 3µl ethidium bromide. Preparation of 10 x FA gel buffer is given in (Table 1)

cDNA PREPERATIONS

cDNAs were synthesized by M-MLV reverse transcriptase (Fermentas, Lithuania) kit. cDNA reaction mixture as follows (Table 2). The total volume of 20µl is incubated for 60 min at 42^{0}C and the reaction is terminated by heating at 70^{0}c for 5 min.

Primers

The following primers were designed after having taken into consideration the Tm value and molecular weight primers has been designed using NCBI tools (Table 3).

Primer Synthesis and reconstitution

The primers for Cytokeratin 20, Cytokeratin 17, Cytokeratin10 and Anillin whose sequences have been given in section3.2.6 were designed and commercially synthesized. The primers were reconstituted in TE buffer at a concentration of 200picomoles/µl, aliquoted into 20picomoles/µl aliquots and frozen at -20^{0}C.

Standardization of PCR conditions with ACTIN

About 1 µl cDNA was taken and PCR was performed in the final volume of 20µl, containing 1X reaction buffer, 10mM dNTP mix, 1.5U Taq polymerase, 20picomoles of primer. The following PCR conditions were followed, initial denaturation at 94^0 C for 4 min, followed by denaturation at 94^0 C for 30 sec, annealing at 60^0 C for 30 sec, extension at72^{0}C for 40 sec, and final extension at72^0 C for 3 min.

PCR for the Biomarkers

cDNAs were synthesized by M-MLV reverse transcriptase (Fermentas, Lithuania) and subjected to polymerase chain reaction (PCR) initially or actin normalization then the specific primers for Anilin,Cytokeratin10,Cytokeratin 17, Cytokeratin 20, were checked for gene expression . PCR conditions for

amplification were: 95°C for 3 min, 95°C for 30sec min and 58°C extension for 30sec and 72°C extension 1min for 30 cycles. The PCR products were visualized using1.2% agarose gel electrophoresis and staining with ethidium bromide

RESULTS

Cell line establishment and maintenance

Biopsy was obtained from healthy donors and washed repeatedly in Antibiotic - Antimycotic (A+A) solution and treated with 0.3% collagenase overnight at 4^{0}C. The cell suspension was then directly seeded onto culture dishes and incubated at 37^{0}C. After sufficient growth, the cells were trypsinized, washed, and expanded or cryopreserved after suspension in freezing medium SW480, HCT 116 and Afibroblasts were grown to confluence , trypsinized ,washed in PBS and used for subsequent RNA isolation.

Table 1. Preparation of 10 X FA gel buffer

200mM	MOPS
50mM	sodium acetate
10mM	EDTA
pHwas adjusted to 7.0 with NaOH	

Table 2. CDNA synthesis reaction protocol

Total RNA	6 µl(1.5ug)
OligodT primer	1µl
DEPC treated water	5µl
5X Reaction buffer	4µl
dNTPS	2µl
RNase Inhibitor	1µl
Reverse Transcriptase	1 µl

RNA extraction

Total RNA was extracted from each of the three cell types (two cancerous and one normal).The extracted RNA was checked on a 1.2%formaldehyde gel (Fig. 1). The results indicate that the total RNA was successfully isolated from the three different cell types and were therefore used to synthesize cDNA.
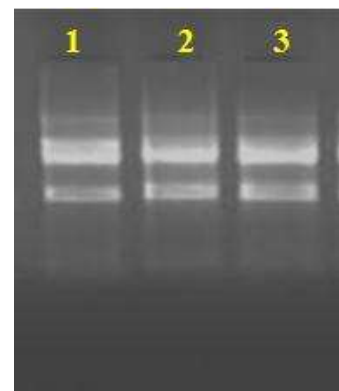

**Fig. 1. Qualitative Analysis of RNA on 1.5% formaldehyde Agarose gel**

Standardization of cDNA synthesis pcr conditions for actin

Total RNA isolated from the 3 cell types, was then used to synthesize cDNA.

Table 3. Primers used for semi qualitative gene expression for the biomarkers includes primer sequences andannealing temperatures

Sl.No	Primer Name	Forward primer	Reverse primer	Annealing temperature Tm
1	Actin	5'TCCATAATGAAGTGTGATGT3'	5'GGACCTGACTCGTCATACTC3'	60^0C
2	Anillin	5'TGGCTAATTGTACCAGTCGTCAGA3'	5'AGGCTTTCCAATAGGTTTGTAGCA3'	58^0C
3	Cytokeratin 10	5'TGGTTCAATGAAAAGAGCAAGGA 3'	5'GGGATTGTTTCAAGGCCAGTT 3'	60^0C
4	Cytokeratin 17	5'CATGCAGGCCTTGGAGATAGA 3'	5'CACGCAGTAGCGGTTCTCTGT 3'	59^0C
5	Cytokeratin 20	5'CAGACACACGGTGAACTATGG3'	5'GATCAGCTTCCACTGTTAGACG3'	60^0C

The synthesized cDNA was used as a template for the standardization of PCR conditions using the actin primers. Amplification (250 bp) was observed (Fig. 2) in all the cell lines under optimized PCR conditions.
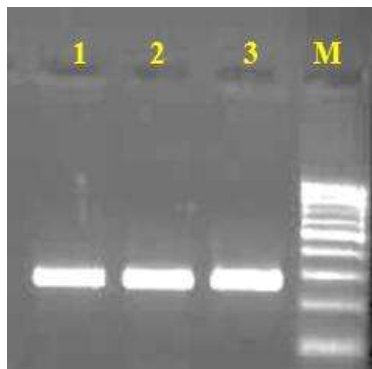
LaneM:100bp marker
Lane1-3-cDNA optimization with β-actin primers (Afibro,SW480,HCT116)

Fig. 2. cDNA optimization for RNA samples
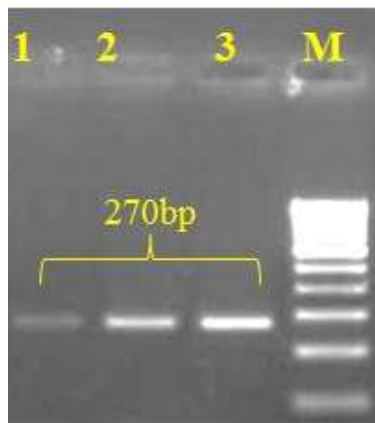
Lane M:100bp marker
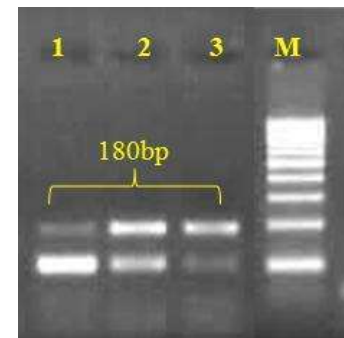Lane1-3-Anilin amplification 270bp (Afibro,SW480,HCT116)
Fig. 3. Semi quantitative analysis of RNA expression using Anilin marker

PCR for the biomarkers

After actin normalization same amount of cDNA was taken then the specific primers for all the three markers was used for expression check an amplification of Anilin(270bp) (Fig 3), Cytokeratin 10(150bp) (Fig 4), Cytokeratin 17(180bp) (Fig5), Cytokeratin 20(370bp) (Fig 6) was observed when PCR products were visualized using1.2% agarose gel electrophoresis.
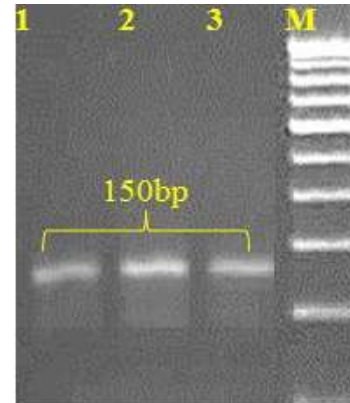
LaneM:100bp marker
Lane1-3-Cytokeratin10amplification150bp (Afibro,SW480,HCT116)
Fig 4. Semi quantitative analysis of RNA expression using Cytokeratin 10 (CK10) marker

LaneM:100bp marker
Lane1-3-Cytokeratin17amplification180bp (Afibro, SW480, HCT116)

Fig. 5. Semi quantitative analysis of RNA expression using Cytokeratin17 (CK17) marker
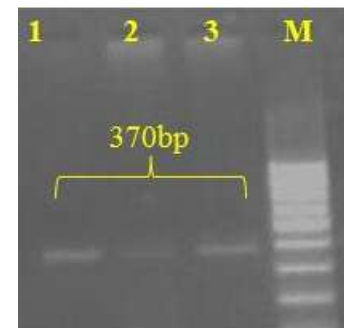
LaneM:100bp marker
Lane1-3-Cytokeratin 20 amplification370bp(HCT116, Afibro,SW480)

Fig. 6. Semi quantitative analysis of RNA expression using Cytokeratin 20(CK20) marker

DISCUSSION

Until superior therapeutic treatments are developed to prevent, treat and cure cancer, the best means of reducing mortality and morbidity in a disease this complex is early detection and diagnosis. In the major solid cancer types such as lung, breast, colon and prostate, long-term survival rates drop precipitously once metastasis has occurred diagnostic measurement of cancer disease progression is essential to successful disease management. For these reasons, development of new and effective biomarkers for cancer detection and diagnosis is central to the cancer problem. Based on literature survey, we identified the markers Cytokeratins 1017, 20 and Anillin as potentially new biomarker candidates which can be used in developing a reliable, sensitive and rapid diagnostic test for screening multiple tumors. Our results indicated that levels of Cytokeratin 17, 20 were higher in both the cancerous cell line compared to the normal cells. Cytokeratin 10 levels were higher in only one of the two cancerous cell lines. This could be because the origin and nature of the two cell line were different, though further tests need to be done to confirm this. If these results prove to be consistent, then there is a possibility of using Cytokeratin 10 as a specific tumor marker for distinguishing between different types of colorectal carcinoma. Our results also show that Anillin levels were also significantly higher in two cancerous cell and therefore can be used as a tumor marker.

Conclusion

In conclusion we have identified Anillin, Cytokeratin17 and Cytokeratin20 as general tumor markers and Cytokeratin 10 as a potential marker for specific types of carcinomas. While these results need to be substantiated, it opens up the possibility of using these markers in conjugation with other known/unknown markers for effective tumor diagnosis. While more and more evidence points toward a clinical relevance for quantification of tumor cells, patient survival will provide the ultimate validation of any proposed marker or combination of marke.

Conflict of interest

Authors declare no conflict of interest

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