

Penetrance of Arsenic Modulates Gene Expression of Epithelial Mesenchymal Transition Marker SOX4, EpCAM and CK19 Genes Through MTHFR C677T Polymorphism Regulation in Circulating Tumour Cells Isolated from Breast Cancer Patients

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Received: 02 Mar 2024

Accepted: 22 Apr 2024

Published: 29 Apr 2024

J Short Name: COO

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Citation:

Saxena AK, Penetrance of Arsenic Modulates Gene Expression Of Epithelial Mesenchymal Transition Marker SOX4, EPCAM and CK19 Genes Through MTHFR C677T Polymorphism Regulation in Circulating Tumour Cells Isolated from Breast Cancer Patients. Clin Onco. 2024; 7(10): 1-4

Keywords:

Arsenic; Epithelial mesenchymal transition marker; MTHFR C677T gene; Circulating tumor cells; Breast cancer

1. Abstract

Arsenic is a common environmental agent that modulates various kind of disease including cancer. However, the exact role of arsenic induce in breast cancer development remains unknown in circulating tumor cells (CTCs). Therefore, present study has been designed with the aims to elucidate the impact of arsenic exposure i.e 24, 48, and 72 hours on epithelial-mesenchymal transition (EMT) markers such as Sox4, EpCAM, and CK19. RT-PCR analysis was carried out for the characterization of EMT with specific primers on agarose gel electrophoresis. DNA copy number variation (DNACNVs) was evaluated to assess genetic susceptibility and MTHFR C677T gene polymorphism was examined to determine the “risk factor” in CTCs isolated from breast cancer patients. Interestingly, findings reveal the differential gene expression and a pattern of down regulation (under expression) were observed during 24 and 48 hours of exposure, followed by up regulation at 72 hours. Genetic heterogeneity of the MTHFR C→T allele was detected only at 48 hours, suggesting that arsenic increase “risk” for developing breast cancer by modulating EMT marker either alone or synergistically with MTHFR in CTCs.

2. Introduction

Arsenic, naturally occurring mutagen exist in ground water and

high concentration is injurious to health (1). The continuous exposure as a source of contaminated drinking water, food preparation and irrigation of crops affecting food chain that may lead to several diseases including cancer due to chromosome aberrations and single strand DNA damage (2). There are large number of basic histopathological and cytological including immunochemistry techniques are documented in the literature whose sensitivity varying up to 60-80% and time taking too. The application of circulating tumor cells (CTCs) for early diagnosis using epithelial mesenchymal transition (EMT) is frontier in cancer medicine, whose efficiency is more than 99% in clinical diagnosis of primary solid tumors (3). Although, the etiopathology of breast cancer and interaction to environmental mutagen (As) with EMT during progression of disease has not been defined satisfactorily in the literature. Therefore, the present study has been designed with the aims to evaluate gene regulation of epithelial cell adhesive molecule (EpCAM) and SRY HMG-BOX transcription factor 4 (Sox4), and cytokeratin 19 (CK19), using in- vitro exposure at different time intervals. DNA copy number variation (DNACNVs) and MTHFR C677T gene polymorphism also determined to assess the genetic susceptibility and risk factor, respectively, in circulating tumor cells isolated from breast cancer patients for early prognostic and diagnostic marker.

3. Material and Methods

Clinically diagnosed cases of breast cancer patients (n=9) and equally age matched controls were used for short term lymphocytes cultures in triplicates along with controls. Present study is approval by the Institutional Ethical Committee (IEC) of All India Institute of Medical Sciences Patna. Blood samples (0.5ml) were collected after informed consent, The cells were exposed with arsenic 0.01ugm/ml (single dose) at different time intervals at 24, 48 and 72 hours, after harvesting, the circulating tumour cells (CTCs) were isolated using Ficoll's gradient methods (4) and total cells were divided in two groups- first cells were fixed in RNazole for RNA isolation followed by gene-expression. In second group, the cells were used for DNA isolation using Promega kit (USA) and quantified by Nanodrop spectrophotometer (Thermo Fisher, USA) and DNA, RNA were store at -80°C, till further study. Polymerase chain reaction (PCR) products of the EpCAM, CK-19 and SOX4 gene expression were characterized by using specific primers (forward & reverse) after confirmation by NCBI Blast. PCR reaction contains 5 µl of 5X Go Taqbuffer, 1 µl of primer set, 1.25 µl dNTP, 0.2 µl TaqDNA polymerase, 50 ng of template DNA and further volume was maintained by nuclease free water. The cycling condition was initial denaturation at 95°C for 5 minutes, denaturation at 95°C for 45s, annealing temperature based on specific target gene as documented in table-1 for 30s and extension at 72°C for 1minute, final extension at 72°C for 7 minutes. PCR products were characterized on agarose gel (1.5%) and individual bands were characterized GelDoc (Bio Red USA). DNACNVs of the EMT marker SOX4, EpCAM and CK19 genes were determined using densitometry analysis of individual

band intensity and compare with controls using inbuilt Image Lab software inside GelDoc (5,6,7). Amplification-refractory mutation system (ARMS-PCR) was used for MTHFR C677T gene polymorphism using tetraplex primer forward/reverse (table-1), assay was performed using SYBR Green and Tm values were calculated to determine genetic heterogeneity between C/ T allele. The cycling conditions consist of initial denaturation at 95°C for 10 min, followed by 30 cycles of amplification steps (95°C for 10 s, 58°C for 10s, 72°C for 10s), final elongation at 72°C and followed by melt-curve analysis as detailed conditions described earlier (8). Melting curves were constructed by lowering the temperature to 65°C and later increasing the temperature by 0.2°C/s to 98°C to measuring the changes in fluorescence intensity.

4. Results

Figures-1, showing the PCR based analysis of differential expression of epithelial mesenchymal transition marker gene expression (Sox4 EpCAM and CK19), after 24, 48 and 72 hours exposure of arsenic. Similarly, bar diagram showing DNACNVs of individual bands using densitometric analysis with controls (red). The gene expression of EpCAM and Sox4 significantly shown down regulated with respect to controls at 24 and 48 hours of arsenic exposure (fig.1AB). Sox4, the early transcription factor also showing a pattern of down-regulation in first 24 to 48 hours and suddenly increased at 72 hours, although the EpCAM and CK19 gene expression also up regulated at 72 hours and reach equal up to controls (fig.1C). MTHFR C677T gene polymorphism showing genetic heterogeneity at 48 hours, where, the Tm values shift from 81.50 (case) to 80.00 GAPDH act as genomic controls.

Table 1: PCR based analysis of epithelial mesenchymal transition markers and methylenetetrahydrofolate reductase primers (forward/reverse) in circulating tumor cells isolated from breast cancer patients

Types of Genes	Forward and Reverse Primer Sequence (5'→3')	Anne. Temp (°C)	References
CK19	F- 5'-ATTCCGCTCCGGGCACCGATCT-3'	60.2	Balducci et al., 2005
	R- 5'-CGCTGATCAGCGCCTGGATATGCG-3'		
MTHFR C677T	F- 5'TGTCATCCCTATTGGCAGGTTACCCCAA-3'	58	Saxena et al., 2016
	R- 5'CCATGTCGGTGCATGCCTCACAAAG-3'		
	C-poly 5'GGCGGGCGGCCGGGAAAAGCTGCGTGATGATGAAATAGG3'		
	T-allele-5'GCACTTGAAGGAGAAGGTGTCTGCGGGCGT-3'		
SOX4	F-5'-GGTCTCTAGTTCTTGCACGCTC-3'	57.2	Zafarnejad et al., 2010
	R-5'-CGGAATCGGCACTAAGGAG-3'		
EpCAM	F-5' ATTCCGCTCCGGGCACCGATCT3'	58.7	Alowaidi et al., 2018
	R-5'CGCTGATCAGCGCCTGGATATGCG3'		

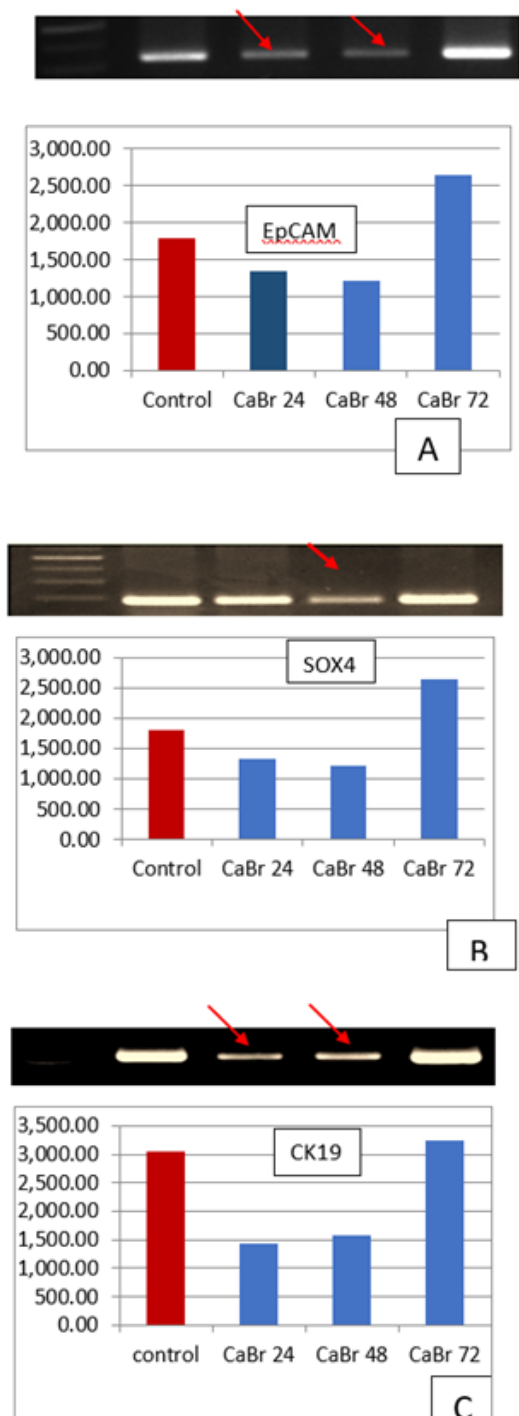


Figure 1A, B & C: In-vitro exposure of arsenic at different time interval 24, 48 &72 hour showing differential expression of epithelial mesenchymal transition expression-CK19 , EpCAM and Sox4 on agarose gel in circulating tumor cells isolated from breast cancer patients. Bar diagram showing DNA CNVs, a pattern of down-regulation in EpCAM and Sox4 were observed at 24 and 48 hours, but CK19 showing significant pattern of down regulation with respect to controls. There is no change were observed after 72 hours exposure in all the three EMT markers.

5. Discussion and Conclusion

In the present study showing the differential expression of EMT marker gene which is modulating perhaps due to heterogeneous

cell -population of breast cancer

Sox4, is early transcription factor 4 (SOX4) marker belongs to gene family of Sry related high mobility group box (SOX-HMG) box region whose gene is mapped on chromosome 6p22.3 with single-exon that encodes protein 47KD comprising of 474 amino acid residues to regulates cellular differentiation and maintenance of growing progenitor cells during malignancy either alone or synergetic manner with transforming growth factor (TGF) mediating signalling [9],The significant increase in over-expression of EpCAM, CK19 and Sox4 gene and DNACNVs at 72 hours, suggesting that involving another path way to recover the damage protein (s) based on antioxidant glutathione-S-transferees gene polymerase by scavengering of free-radicals and nullify the action of toxin (arsenic) on CTCs in breast cancer patients. CK19 is associated in variety of tumour and or over-expression (mRNA) was observed in primary and metastatic lesion of breast cancer patients [10-11]. EpCAM, a transmembrane cell adhesion protein and over-expression is observed in both breast as well as ovarian cancer [12-113]. Earlier study of the same author is the evident that differential expression of Oct4, Nanog and Sox2 genes fail to maintain pluripotency during organogenesis (14). Study of CTCs in pancreatic tumors also reveals that genetic heterogeneity of MTHFR C677T gene polymorphism (15,16, 17).

6. Conclusion

Interestingly, present study showing genetic heterogeneity at 48 hours due to substitution of nucleotide (point mutation) from cysteine to thymidine followed by change in amino acid from alanine to valine, confirming may be due to accumulation of arsenic in first 24 hours of the cell-cycle and toxic effect on folate metabolism was observed at 48 hours in CTCs, suggesting increasing “risk factor” for breast cancer patients. These finding also supported by DNACNVs to increase genetic susceptibility either with MTHFR or EMT gene expression, hence, timely management is required through early diagnosis and becomes an essential to help for the clinicians.

7. Acknowledgement

AKS is thankfully acknowledged to the Executive Director, AIIMS Patna, for providing valuable suggestions. We also extend our thanks to the Department of Science and Technology (DST), New Delhi for providing financial assistance and patients who participated in this study.

8. Ethics Approval

The study was approved by Institute ethical committee (IEC) of AIIMS Patna (AIIMS/Pat/IRC/2020/610).

9. Conflict of Interest

There is no conflict of interest between the authors

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