



Research Article

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DNA Copy Number Variation of Sox4 Fail to Maintain Pluripotency Results to Promote Metastasis in Breast Cancer Patients

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Abstract

Sox4, early transcription factor and is play an important role during movement of epithelial to mesenchymal transition through invasion and followed by initiation of tumor progression in Breast Cancer (BC) patients. Genetic diversity of stem cell are the unique phenomenon and has been associated with epigenetic modification either alone or synergistic manner with other stem cell markers like Oct4 or Nanog to maintain pluripotency during differentiation of tumor cells and how to maintain pluripotency during progression of disease in cancer patients has been poorly defined. Hence, present study has been designed with aim to evaluate the percentage (%) frequency of DNA Copy Number Variations (DNACNVs) of Sox4 gene expression and compared with Oct4 and Nanog , after isolation of genomic DNA followed by Polymerase Chain Reaction (PCR) with specific forward /reverse primers for characterization individual bands on agarose (1.5%) gel electrophoresis after ethidium bromide staining . Densitometric analysis were carried out of individual band of all the three stem cell markers using in build software from Gel doc system. Sox4 bar diagram showing a pattern, where DNACNVs increase and then decrease with respect to controls and statistical analysis showing the significant difference ($p < 0.01$), suggesting, that strong signalling is required to maintain synergism with Nanog might be responsible to promotes metastasis. Oct4 expression fail to support to maintain pluripotency because of poor-signalling resulting initiation of metastasis. Hence, Sox4 could be used as preventive, prognostic and diagnostic biomarker during early genetic screening for the cancer patients followed by the data also support to the clinicians that stem cells may help for better management as a source of regenerative medicine.

Keywords: Breast Cancer; Stem Cell; Sox4; Oct4; Nanog; Pluripotency.

Introduction

Globally, Breast Cancer (BC) is one of the most common disease associated with women health due to poor early prognosis and diagnosis. Genetic and epigenetic factors make the etiopathology of BC more complex due to variable expression of Epithelial - Mesenchymal Transition (EMT) markers-Sox4, EpCAM and CK19 in Circulating Tumor Cells (CTCs) isolated from variety of tumors during progression of disease [1-4]. Sox4 gene belong to Sry-related High Mobility-Group (HMG) domain and act as early transcription

factor to regulate cellular - differentiation process and to decide the fate of tumours during metastasis [5]. Sox4 gene assigned on chromosome- 6p22.3 with single exon that encodes 47KD protein comprising of 474 amino acids residues regulating growing progenitor cells through multiple signalling, among Transforming Growth Factors (TGF) or Protein Kinase (PKC) are responsible for the activation of oncogene (KRAS). Sox4 induces aggressive behaviour after prolonged exposure during transition of epithelial cells by TGF β signalling in differentiating tumor cells [6,7].

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During tumorigenesis, Sox4 may also act as potential biomarkers for early diagnosis and their gene-expression varying with different age and ethnic groups depending upon invasion to neighbouring tissues (organs) and migration of CTCs [8,9]. Nanog, another transcription factor, and is essential to maintain pluripotency with variable expression suppresses the differentiation of embryonic stem cells due to self-renewal property during angiogenesis [10]. Hence, the curiosity has been developed with the aim to explore the functional etiopathology of Sox4 maintaining pluripotency either alone or in synergistic fashion either with Oct4 or Nanog and also evaluate the frequency of DNACNVs in individual cancer patients to explore the genetic susceptibility. The present study is small but have great potential to the clinicians for early prognosis, diagnosis and followed by timely management using stem cells targeted therapeutics as a source of regenerative medicine.

Materials and Methods

Present study includes clinically diagnosed cases (n=38) of breast cancer patients and controls (n=38) were used for the present study. The study is approved by Institute Ethical Committee (IEC), All India Institute of Medical Sciences Patna (Bihar). Blood samples (1.0 ml) were collected in a EDTA vial after consent from the patients. Genomic DNA was isolated using kit protocol and half of the sample (cells) were fixed in RNAzole for isolation of RNA for quantification by nanodrop spectrophotometer. The samples were stored at -20°C, till further analysis of stem cells markers using PCR technique with specific forward and reverse primers on agarose gel (1.5%) electrophoresis.

Characterization of Sox4, Oct4 and Nanog.

Table-1 showing the PCR based strategy for Sox4, Oct4 and Nanog analysis along with their annealing temperature using specific primers. The nucleotide sequences of the primers further confirm by NCBI-BLAST (<http://blast.ncbi.nlm.nih.gov>). Total volume (25 µl) of PCR was achieved in reaction mixture containing 5X Green GoTaq PCR reaction buffer, dNTPs (10 mM), 1 µl each of 10 pmol of Oct4, Nanog, Sox2 and Sox4 primer (forward & reverse), 0.2 µl of GoTaq DNA polymerase (5 U/µl) and 50 ng DNA in thermocycler (Agilent Technologies, Sure Cyclor 8800). Reaction summary was carried out in 35 cycles comprising, hot start at 95°C for 5 minutes, denaturation at 95°C for 30 seconds, annealing at 57.2°C for 30 seconds, elongation at 72°C for 30 seconds, followed by final elongation at 72°C for 8 minutes. The amplified PCR product of the candidate genes were characterized individually on agarose gel electrophoresis after ethidium bromide staining. The individual band intensity was measured by densitometry to evaluates the frequency of DNACNVs using Image Lab inbuilt Software of Gel Doc system.

Table 1: PCR strategy was used for the characterization of stem cell marker in breast cancer patients.

Stem cell Marker	Forward & Reverse Primer Sequences (5' → 3')	Annealing Temp (°C)	References
Sox4	f5'-GGTCTCTAGTTCTGCACGCTC-3'	57.2	Jafarnejad, et al (9)
Sox4	r5'-CGGAATCGGCACTAAGGAG-3'		
Oct4	f5-'GACCATCTGCCGCTTTGAG-3'	60.0	Henderson, et al (11)
Oct4	r5;-CCCCCTGCCCCATTCTTA-3'		
Nanog3	f5'-CTGTGATTTGTGGGCTG AA-3'	56.0	Nettersheim, et al (10)
Nanog3	r5'-TGTTTGCTTTGGGACTGGT-3'		

Table 2: Statistical analysis showing the frequency (%), O.R and C.I for Oct4, Nanog and Sox4 in breast cancer patients with controls.

Type of genes	Mean ± SD	Gene expression	Frequency (%) cases controls		C.I. at 95% max min		OR	p-value
Sox 4	2,127.3 ± 368.6	Over-expression	6 (75.0)	1 (14.3)	5.11	0.53	18.0	0.018*
	1618.9 ± 216.0	Under-expression	2 (25.0)	6 (85.7)	11.42	0.15		
Oct-4	2099.0 ± 106.3	Over-expression	6 (75.0)	4 (57.1)	33.62	2.68	2.25	0.88
	2108.5 ± 134.5	Under-expression	2 (25.0)	3 (42.9)	7.7	1.59		
Nanog	2191.3 ± 223.1	Over expression	4 (50.0)	4 (57.1)	8.46	0.529	0.75	0.001*
	1678.5 ± 265.9	Under-expression	4 (50.0)	3 (42.9)	4.93	0.474		

DNA Copy Number Variations in Sox4, Oct4 and Nanog

Fluorescence stain, ethidium bromide was used for the visualization of individual bands intensity and densitometry analysis were carried for three stem cell markers- Sox4, Oct and Nanog in BC patients and compare with age match controls using Image lab software 5.1 of inbuilt Gel Doc system (Bio Rad USA). Data was evaluated in the terms of either over-expression (up regulation) or under-expression (down regulation) and bar diagram was constructed for visualization of the frequency distribution of DNACNVs of stem cell markers to determine the genetic susceptibility during advancement of disease in cancer patients.

Statistical analysis

Chi-square test (two tailed) was used for evaluation significant (p-value) difference between cases and controls. Data was also calculated mean ± standard deviation, standard error, Odd-Ratio (O.R) and Confidence Intervals (C.I) at 95% (max & min) values to find the interaction between two variables.

Results

Table-2 showing the detail analysis of the data of three pluripotent stem cell markers - Sox4, Oct4 and Nanog along with the frequency of DNACNVs in BC patients. Interestingly, Sox4 showing significant over-expression with respect to controls as shown in Figure 1C & 1D. The calculated values of mean±SD (2127.3±36.8),

confidence interval C.I) varies between 511-0.53 with odd ratio 18.0 (Table 2). Similarly, bar diagram of DNACNVs showing over-expression in Oct4 and Nanog with calculated mean±SD values 2099.0±106.3, 2191.3±223.1, confidence interval varying between 33.62-2.68, 8.46-0.5 with O.R 2.25, 0.75, respectively as depicted in Figure 1A,B,E&F(arrow). Interestingly, Nanog showing again highly significant ($p<0.001$) differences (over-expression) with calculated value of O.R is 0.75 and in C.I at 95% intervals that varying between 8.46 to 0.529 with respect to controls.

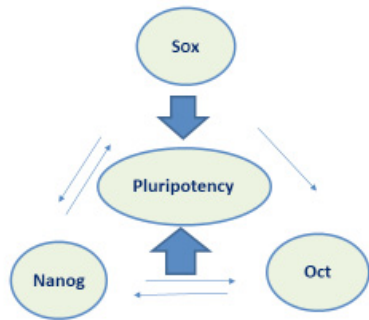


Figure 1: Flow chart showing gene regulation of Stem cell marker Sox4, Nanog and Oct4 in breast cancer patients fail to maintain pluripotency. Synergism (double line arrow) showing significant positive relationship between Sox4 and Nanog, but Oct4 (single line arrow) fail to maintain relationship resulting Sox4 shows aggressiveness in phenotypic changes during transition from epithelial to mesenchymal cells and induce metastasis.

Discussion

Stem cell biology is the frontier area of regenerative medicine and are known to participate in cellular-differentiation, invasion and migration in variety of diseases other than tumorigenesis [12]. Etiopathology of stem cells (progenitor cell) with functional aspects has been poorly documented in literature and antitumor agent or environmental mutagen (arsenic) increase risk of developing cancer after genetic and epigenetic modification [13]. Present study is interesting and significant finding in DNA copy number of Sox4 confirm to induce metastasis during tumorigenesis. In earlier study of the same group emphasize that stem cells like Oct4, Nanog and Sox2 play a relevant role in normal differentiation of germ-cell and their expression maintain “central- dogma” of pluripotency during organogenesis other than cancer [14,15]. In fact, Sox proteins act as architect to decide the fate of early developmental processes including sex-determination, neural , chondrogenesis and cardiac development [16,17]. Zhang et al. also suggested that Sox4 expression is also influence by chemokine transmembrane receptor [8]. The most interesting part of the present study showing significant relationship of Sox4 between DNACNVs and over-expression that confirming the growth, (proliferation), migration and invasion cells to induce metastasis BC patients.

Sox-4, an early transcription factor and is regulatory molecule during developmental process through binding to the adjacent site of the DNA with other partner for activation or repression, that confirm association with Nanog, might be the “good” partner for maintain pluripotency in BC patients [12]. The ectopic expression of Sox4 increase the genetic susceptibility after using Paclitaxel (PTX) or Chemokine Transmembrane Receptor-7 (CXCR7) pathway to decrease metastasis [8]. During Transition of Epithelial to Mesenchymal cells (EMT) involves another regulatory pathway that includes CK19, EpCAM and Sox4, and modulates gene-expression through TGF β in synchronize manner, suggesting induce tissues specific genetic susceptibility during progression of disease [1,18]. Over-expression of Sox4 is responsible for aggressive behavior during transition of epithelial to mesenchymal cells through EpCAM or CK19 in liver and pancreatic tumor [19-20]. Sox4 mRNA expression is dependent on TGF- β , a pleiotrophic cytokine has been associated with glioma and pituitary cells through Sox2, suggesting multilineage differentiation quality of stem cells [21,22]. Data of the present study confirm that functional activity of Sox4 is not alone but responsible with synergistic fashion with Nanog together inhibit metastasis in during progression of disease. Although, over-expression of Nanog as transcription factor becomes relevant for differentiation of tumor cells, but exact mechanism how to accelerate mammary tumorigenesis is still lacking [23]. Present study of DNACNVs also show over-expression of Oct4, but showing lacking significance difference with respect to controls, suggesting fail to form complex either with Sox4 or Nanog resulting catastrophe regulation in maintaining pluripotency. The mimicry (up/down regulation) of stem cell markers further confirmed by DNACNVs in individual patients occurs either due to different stages of malignancy or varying genetic susceptibility. Another aspect of the variation of DANCNVs is genetic heterogeneity, might be another aspect for unequal proliferation of circulating tumor cells during migration, invasion to the neighbouring tissues fail to maintain pluripotency in BC patients.

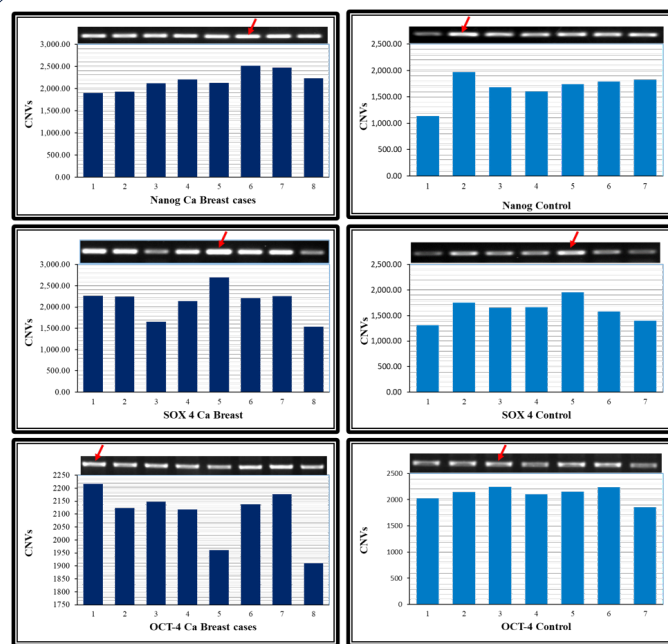


Figure 2: PCR based analysis for the characterization of stem cell markers- Nanog, Sox4 and Oct4 on agarose (1.5) gel electrophoresis, after staining with ethidium bromide using specific forward & reverse primers in BC patients. Bar diagrams showing the frequency of DNACNVs (dark blue) of individual patients and compare with controls (light blue). Nanog showing over-expression in case 5,6 & 7 as compared to controls (A&B). Sox4 showing significant systemic pattern of increase-expression in case 3 to 4 and reach maximum in case 5 and then down regulated in case 6,7& 8 (C&D). Similarly, Oct4 showing significant decrease in case 5 & 8 with respect to controls (E&F), respectively.

Conclusion

Tissue culture engineering based on stem cell tumour biology has great value for the clinicians in therapeutics as a source of regenerative medicine in variety of disease like chronic wound healing and early management of cancer. In order to develop effective treatment approach, there is an insistent need to comprehend the causes of heterogeneity in cancer patients with its cellular and molecular traits are as follow - 1) the functional activity of Sox4 confirm that act as synergistic fashion with Nanog, 2) significant variation of DNACNVs increases genetic susceptibility due to different stages of cancer either pre or post metastatic, and 3) The aggressive behaviour of epithelial to mesenchymal cell confirm by DNACNVs of Sox4 concluding that stem cell therapy act as source of regenerative medicine may help for cancer patients as supportive tool without any side effects.

Declarations

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Authors' contributions: AKS executed experimental design, implementation and proof reading.

Ethics approval and consent to participate: Study was approved by Institute ethical committee of AIIMS Patna (AIIMS/Pat/IRC/2020/610), and informed consent form was dually signed by patients.

Patient consent for publication: Patients consent form was dually signed by patients involved in study.

Competing interests: There are no competing interests among the authors.

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