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Research Article

Clinico-Pathological Correlates of MCM5 and FOXM1 Expression in Primary Breast Cancer Tissues

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Abstract

Background

Breast cancer is one of the leading causes of cancer-related mortality; meanwhile, it can be curable when detected at earlier stages; thus, retrieving breast cancer biomarkers with adequate sensitivity and specificity for early detection is an urgent need.

Methods

Bioinformatics tools were used to retrieve Mini-chromosome Maintenance Complex component 5(MCM5)and its highly correlated geneForkhead box M1 (FOXM1); based on previous microarray studies from public breast cancer databases; the expression of both genes has been evaluated in breast malignant lesions, benign lesions and normal breast tissue bysemi quantitative RT-PCR.

Results

A significant difference was observed in the Positivity rate of MCM5 and FOXM1mRNA in malignant group i.e.(76.3%,81.3%)as compared with both benign group (21.1%,15.6%)and healthy normal group (2.6%,3.1%) respectively (P=0.000). The combined sensitivity of MCM5 and FOXM1mRNA by either qualitative or semiquantitative RT-PCR in early stage (0+1) breast cancer was 100%, While their combined specificities were 64.8% and 78.3%, respectively. The combined sensitivity of MCM5 and FOXM1mRNA by either qualitative or semi quantitative RT-PCR in Low Grade Breast Cancer was 100%, while their combined specificities were 64.8% and 78.3%, respectively.

Conclusions

MCM5 and FOXM1are two novel biomarkers that may be exploited to improve breast cancer early detection as well as therapeutic targeting. Further studies are warranted in these directions.

Keywords: Breast cancer; MCM5; FOXM1; Bioinformatics

Introduction

Breast cancer is by far the most common cancer among women indeveloped and developing countries; accounting for 22.9% of all female cancers [1]. It is also the leading cause of cancer death in females. Earlier detection and treatment are thought to improve outcomes, yet even very small lesions at the limit of detection by mammography, magnetic resonance imaging, or palpation can progress to metastatic disease [2].

A large number of molecules have been evaluated as potential prognostic/ predictive factors of breast cancer. Well established

prognostic factors in breast cancer include ki-67, estrogen receptor, progesterone receptor and HER-2 neu. Other investigational prognostic factors include apoptosis-related proteins, cell cycle molecules, plasminogen activators/ inhibitors and angiogenesis-related proteins [3].

More and more, the discovery of relevant biomarkers is aided by in silico techniques based on implementing computational chemistry and data mining on large molecular databases. However, database searching is an even larger source of valuable information that can potentially be utilized [4].

Some of the genes expressed in complex diseases (like cancer) correspond directly to the disease phenotype, (sometimes called driver genes), while others represent closely-related first-degree neighbors in gene interaction space. The remaining genes consist of genes that are often not causally related to the disease. For prognostic and diagnostic purposes, it is vital to be able to segregate the group of "driver" genes and their first-degree neighbors [5].

The mammary gland is a dynamic organ that undergoes continuous cycles of proliferation and apoptosis between puberty, pregnancy, lactation and menopause. A clear understanding of mammary progenitor regulation and the process by which these cells become differentiated has profound implications in the field of breast cancer [6].

The Mini-chromosome maintenance complex (MCM 2-7) proteins are present in the proliferative phases of the cell cycle but are absent in the quiescent, terminally differentiated and senescent out-of-cycle states [7]. MCMs expression in different human cancer tumors has recently been the focus of extensive research [8].

MCM proteins play vital role in DNA replication, they are related to cell proliferation, and serve as useful markers for cancer screening and prognosis. They are encoded by genes which are parts of the MCM genes from MCM 2-7 [9].

Moreover, MCM5 has vital role also in transcription regulation, as MCM3-MCM5 interacts with the transcription factor (STAT1 alpha iso-form) [10]. Another study showed that the MCM5is required for transcription elongation of MRNA Pol II [11].

Mammalian transcription factor Forkhead Box M1 (FOXM1) is a member of the family of Forkhead transcription factors which is characterized by an evolutionarily conserved DNA binding domain called Forkhead or winged-helix domain [12].

FOXM1 expression correlates with the proliferative state of the cell. Expression of FOXM1 is negatively regulated in quiescent or terminally-differentiated cells. Meanwhile it is specifically expressed in proliferating cells [13]. Elevated expression of FOXM1 is also observed in a multitude of solid malignancies [14].

FOXM1 regulates a variety of processes in mammalian cells through regulating the transcription of genes important for cell cycle progression, cell proliferation and survival, DNA damage repair, angiogenesis and chemotherapeutic drug response [15].

Starting in 2002, FOXM1 was labeled an oncoprotein, since then, researchers have linked FOXM1 over expression to almost all types of human cancers; however, till the moment this information was not exploited for diagnostic or therapeutic purposes [15].

Overall, there is evidence pointing to FOXM1deregulation as a major cause of carcinogenesis and therapy resistance, suggesting that targeting FOXM1 activity in malignant cells could be a promising strategy for cancer treatment [16].

The core of the present study was to evaluate the tissue expression of both MCM5, 1FOX Mgenes in relation to clinicopathological factors of breast cancer and to explore their synergistic expression.

Materials and Methods

Patient's population

This pilot study was conducted on 54 Egyptian female patients who were diagnosed with breast cancer and underwent curative surgery at General Surgery department, Ain Shams University Hospitals and 20 healthy normal volunteers with matching age and sex to the patients' groups who underwent plastic breast reduction surgery. The study was performed inaccordance with Declaration of Helsinki and was approved by the Research Ethics Committee of Ain Shams University, Cairo, Egypt. An informed consent was obtained fromall patients. Clinical staging of breast cancer was performed according to TNM classification American Joint Committee on Cancer. AJCC, 2010 [17] and graded according to American Cancer Society, 2014 [18] ER, PR and Her-2 neu Scores were detected by an experienced pathologist using immunohistochemistry techniques.

Subjects were divided into the following groups

Group A: Malignant breast cases (n=37, of mean age 53.4±14.7 years, median 55 years and range from 20-81 years); Regarding their Stages: they included 16 cases of stage I, 16 cases of stage II and 5 cases of stage III, Regarding the Grade: they included 9 cases of grade 1, 25 cases of grade 2 and 3 cases of grade 3.

Group B: Benign cases diagnosed as fibro adenoma(n=17, of mean age 48.4±13.8 years, median 52 years and range from 20-63 years).

Group C: Healthy normal individuals after breast reduction surgery (n=20, of mean age 51.6±11.1 years, median 49.5years, and range from 36-77 years).

Biomarker identification and verification through bioinformatics analysis

- We used bioinformatics tools in order to retrieve multiple genes that are mechanistically linked to each other and to breast cancer pathways or functional networks; MCM5 and its highly correlated gene FOXM1 based on previous microarray studies. Such in silicodata is based on previous microarray studies that integrated both, the previous information gained from gene expression profiling and the microarray gene expression profiling of protein-coding genes.
- This step included biomarker retrieval from breast cancer databases; Genes to System Breast cancer database [19] Available at http://www.itb.cnr.it/breastcancer/and Expression Atlas database, Available at http://www.ebi.ac.uk/gxa/home followed by biomarker verification through pathway enrichment analysis through KEGG pathway. Finally, biomarker validation through prioritizations of supposed disease genes, supported by functional hypotheses [20].

Biomarker validation

MCM5 and FOXM1 were evaluated with RT PCR (Reverse transcription polymerase chain reaction)in the breast tissue samples to validate their diagnostic and prognostic value for breast cancer.

Extraction of totalMRNA from breast tissue samples

Total MRNA was extracted from breast tissue using Qiazol kit (QIAGEN, USA).

Detection of MCM5 and 1FOXMby semi-qualitative RT-PCR

The primers for MCM5 and 1FOX Mamplification were checked using UCSC genome browser at http://genome.ucsc.edu/cgi-bin/hgBlat, followed by checking if the amplified fragment has any homology with other genomic regions (using NCBI BLAST nucleotide search function) tested and did not show high complementarily to any other DNA sequence listed with NCBI. The primers were purchased from Metabionintem RNAtional AG).

Reverse transcription-polymerase chain reaction (RT PCR)

Extracted total mRNA was used for the detection of MCM5 and FOXM1 mRNA by qualitative and semi quantitative T-PCR. RT-PCRs were performed by using QIAGEN, USA.One Step RT-PCR Enzyme Mix which contains especially formulated enzyme blend specific for both reverse transcription and PCR amplification. The first step of RT was performed at 50˚C for 30 minutes, and then cDNA was amplified to detect MCM5 and FOXM1 using gene specific primers (Table 1). The PCR conditions for both genes were optimized in Hybaid thermal cycler Thermo Electron (formerly Hybaid) Waltham, MA, USA) as follows: First step of RT at 50^0C for 30 minutes, theinitial melting at 95^0C for 5 min; 35 cycles of 94^0Cfor 1 min; 58^0C in case of MCM5 & 56oC in case of FOXM1 (for 1 min); 72^0C for 1 min; and final extension at 72^0C for 5 min. The amplified cDNA of 172 base pair in case of MCM5 & 370 base pair in case of FOXM1 was separated and visualized on ethidiumbromide-stained 2 % agarose gel electrophoresis (Figure 1).

Gene	Sequence	Temperature	References
MCM5 Forward Reverse	 5`CCC ATT GGG GTA TAC ACG TC-3` 5`CAC GGT CAT CTT CTC GCA TCT-3`	 60^0C 61^0C	e-PCRat http://www.ncbi.nlm.nih.gov/
FOXM1 Forward Reverse	 5`CAC CCC AGT GCC AAC CGC TAC TTG-3` 5`AAA GAG GAG CTA TCC CCT CCT CAG-3`	 70^0C 67^0C	[14]
Beta actin Forward Reverse	 5'-CTA CGT CGC CCT GGA CTT CGA GC -3' 5'-GAT GGA GCC GCC GAT CCA CAC GG-3'	 67^0C 69^0C	[21]

Table 1: Gene-specific RT PCR assay.

For semi-quantitative analysis of MCM5 and FOXM1 MRNA, expression of b-action in each sample was determined as an in them RNA control for reaction efficiency and to normalize for sample to sample variation in mRNA amount. Thus, control reactions were amplified using the b-actin-specific primers shown in table-1 with the generation of a 385-bp fragment. The signal intensities in agarose gel of MCM5 and FOXM1mRNA in each sample were determined relative tothat of b-action in the same sample using ''Quantity one 'computer program version 4.6.3, Bio-Rad Laboratories, USA, thus determining the relative amount of different samples.

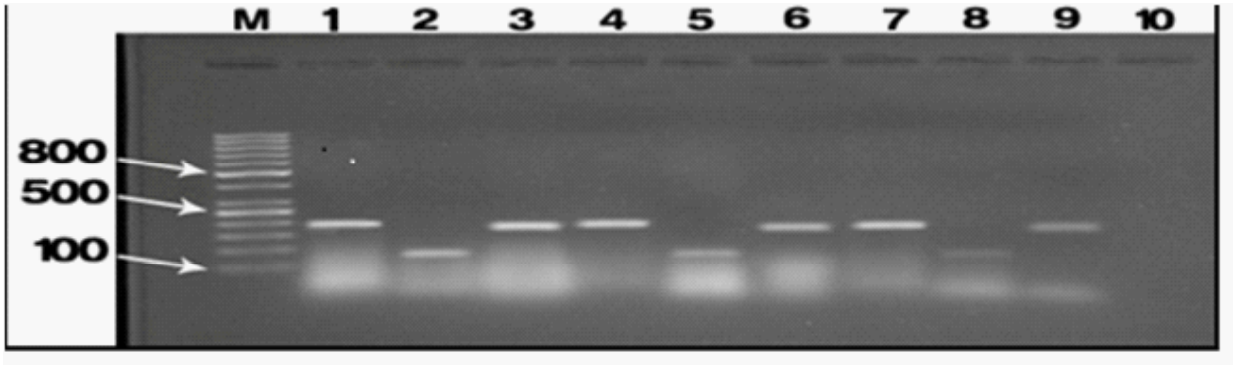

Figure 1: Ethidium Bromide-Stained Agarose Gel Electrophoresis showing the amplified RT-PCR products of β-actin (385 bp)lanes 1,4,7 / MCM5 (172 bp) lanes 2,5,8 / FOXM1 (370bp) lanes 3,6,9 from breast tissues. M=DNA 100 bp ladder, Lane 10 = negative control. (Lanes 1,2,3 = Breast cancer, Lanes 4,5,6 = Benign breast lesion, Lanes 7,8,9,=Normal breast tissue).

Statistical analysis

Unvariate analyses were performed using a chi-square test of association of categorical variables. The threshold value for optimal sensitivity and specificity of MCM5 and FOXM1mRNA was determined by Receiver-Operating Characteristics (ROC) curve. The nonparametric Krausakul Wallis Test was used for the statistical comparison of variables among groups. Sensitivity, specificity, Positive Predictive Value (PPV), Negative Predictive Value(NPV), and accuracy were calculated according to standard statistical methods. All analyses were performed using Statistical Package for the Social Sciences software (SPSS17Inc. Chicago, IL).

Results

Baseline patient characteristics

The different clinic pathological factors were compared in different groups. The differences were significant for family history, BMI, using Oral Contraceptive Drugs (OCT) and hormonal therapy in the past, ER, PR and Her-2 positive patient between malignant, benign and healthy normal groups(P< 0.01) (Table: 2).

Clinicopathological factors		Group			
					2χ
		Malignant	Benign	Healthy normal	(P)
		no. (%)	no. (%)	no. (%)	
Parity	Nullipara (10)	6 (16.2%)	2 (11.8%)	2 (10%)	0.487
					p:NS= (0.784)
	Mutipara (64)	31 (83.8%)	15 (88.2%)	18 (90%)	
Menopausal	Premenopausal (28)	13 (35.1%)	6 (35.3%)	9 (45%)	0.598
	Postmenopausal (46)	24 (64.9%)	11 (64.7%)	11 (55%)	p:NS= (0.742)
Family history	Positive (16)	15 (40.5%)	1 (5.9%)	0 (0%)	15.817
					p: S=(0.000)**
	Negative (58)	22 (59.5%)	16 (94.1%)	20 (100%)	
BMI	Normal (30)	8 (21.6%)	9 (52.9%)	13 (65%)	20.186
	Overweight (24)	11 (29.7%)	7 (41.2%)	6 (30%)	p: S=(0.000)**
	Obese (20)	18 (48.6%)	1 (5.9%)	1 (5%)	
OCT	Past administration (35)	22 (59.5%)	4 (23.5%)	9 (45%)	6.091
					p:S= (0.048)*

	Never (39)	15 (40.5%)	13 (76.5%)	11 (55%)	
HT	Past administration (30)	22 (59.5%)	5 (29.4%)	3 (15%)	11.78
					p: S=(0.003)**
	Never (44)	15 (40.5%)	12 (70.6%)	17 (85%)	
ER	Positive (20)	20 (54.1%)	0 (0%)	0 (0%)	27.407
					p:S= (0.000)**
	Negative (54)	17(45.9%)	17 (100%)	20 (100%)	
PR	Positive (21)	21 (56.8%)	0 (0%)	0 (0%)	29.321
					p:S= (0.000)**
	Negative (53)	16 (43.2%)	17 (100%)	20 (100%)	
Her-2neu	Positive (8)	8 (21.6%)	0 (0%)	0 (0%)	8.970 p:S= (0.011)**
	Negative (66)	29 (78.4%)	17 (100%)	20 (100%)	

Table 2: Clinicopathological Factors of Different Groups Of The study.

As regards Histopathological characteristics of the Malignant Group, (64.9%) IDC, (24.3%) mixed IDC and ILC and 10.8% were other special types eg: Adenocarcinoma, inflammatory breast disease and Paget disease of the breast. Regarding tumor grades; (24.3%) were grade1, (67.6%) were grade 2 and (8.1%)were grade 3. Concerning the tumor stages (43.2%) were stage (I) (43.2%) were stage II and (13.5%) were late stage (III). The percentage of molecular subtypes of breast cancer were (40.5%), (21.6%), (27%) and (10.8%) with Luminal A, Luminal B, Basal and Her-2 neuover expressing Subtype respectively.

ER, PR and Her-2 neustatus was examined in all studied groups, using Chi-square analysis. In the malignant group the overall sensitivity of ER, PR and Her-2 neu were (54.1%, 56.8% and 21.6% respectively), Normal groups the sensitivity of ER, PR and Her-2-neu were 0 %(Table 3).

	ER			**PR**			**Her neu**		
	Positive	**Negative**	χ^2 **(P)**	**Positive**	**Negative**	χ^2 **(P)**	**positive**	**Negative**	χ^2 **(P)**
Malignant	20	17	27.407	21	16	29.321	8	29	8.970 p:S= (0.011)**
	54.10%	45.90%	p:S= (0.000)**	56.80%	43.20%	p:S= (0.000)**	21.60%	78.40%	
Benign	0	17		0	17		0	17	
	0%	100%		0%	100%	0%	100%		
Normal	0	20		0	20		0	20	
	0%	100%		0%	100%		0%	10%	
Chi- square test **p (< 0.01): highly significant									

Table 3: The Positivity rates of ER, PR and Her-2neu in Different Groups of the Study.

MCM5 and FOXM1 expression in the overall population (Figure 2, 3)

MCM5 expression was statistically analyzed in all studied groups, using Chi-square analysis, the overall sensitivities of MCM5 were (76.3% 21.1% and 2.6% respectively) of malignant, benign and normal cases respectively as shown in Table 4.

	MCM5 expression		
	Positive	Negative	χ^2 (P)
Malignant (37)	29	8	28.144
	76.30%	22.20%	p:S=(0.000)**

Benign (17)	8	9	
	21.10%	25%	
Normal (20)	1	19	
	2.60%	52.80%	
**p < 0.01: is highly significant.			

Table 4: MCM5 expression in Breast Tissues examined by RT-PCR among Different Groups of the Study.

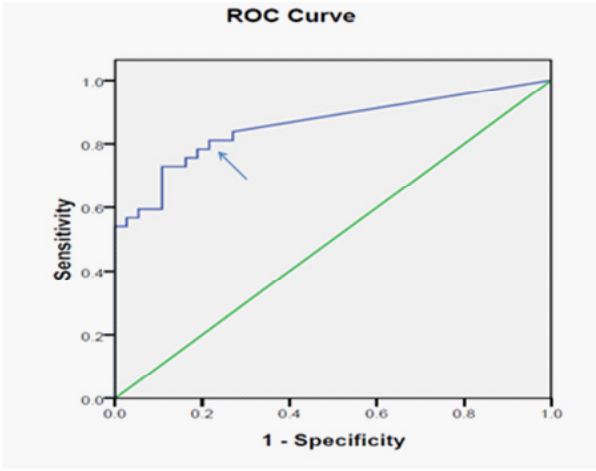

Figure 2: ROC curve of MCM5, the best cutoff point for MCM5 MRNA was 0.865, sensitivity =78.3, specificity = % 75.6%.

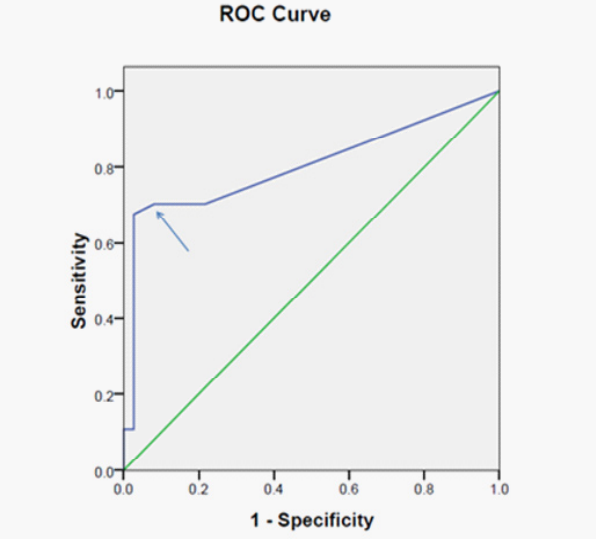

Figure 3: ROC curve of FOXM1, The best cutoff point for FOXM1MRNA was 0.99, sensitivity = 70.2%, specificity = 83.7%

FOXM1 expression was statistically analyzed in all studied groups, using Chi-square analysis, the overall sensitivities of FOXM1 expression were (81.3%, 15.6% and 3.1% respectively)of malignant, benign and normal group respectively, while (26.2%, 28.6% and 45.2%) was negative in malignant, benign and normal group respectively as shown in Table5.

	FOXM1expression		
	Positive	Negative	χ^2 (P)
Malignant (37)	26	11	24.255
	81.30%	26.20%	p:S=(0.000)**
Benign (17)	5	12	
	15.60%	28.60%	
Normal (20)	1	19	
	3.10%	45.20%	
**p < 0.01: is highly significant			

Table 5: FOXM1 expression in Breast Tissues examined by RT-PCR among different Groups of the study.

MCM5 and FOXM1 expression in early stage disease

The overall sensitivities of MCM5 and FOXM1mRNA by either qualitative or semi quantitative RT-PCR in early stage 0+10 Breast Cancer were 87.5% and 62.5% respectively. Furthermore the specificity increased after semi quantization of MCM5, FOXM1mRNA in early stage from 75.6% and 83.7% to 81% and 91.8% respectively.

The combined sensitivity of MCM5 and 1FOXMmRNA by either qualitative orsemi quantitative in early stage (0+1) Breast Cancer was 100%, While their combined specificities were 64.8% and 78.3%, respectively (Table6-9).

Group	*Semi quantitative MCM5 by RT PCRa	No. of cases › .865(%)b
Normal control:		
Median	0.002	0/20.(0%)
Range	.0284_.0879	
Mean Ranks	17.9	
Benign:		
Median	0.712	7/17(41.2%)
Range	.2970_.9772	
Mean Ranks	31.76	
Malignant:		

Median	1.6418	
Range	1.1804_1.7524	29/37 (78.4%)
Mean Ranks	50.73	
X^2	34.922	32.418
P	0.000**	0.000**

*Normalized qtyis the trace quantity of the MCM5 band (average intensity x mm2)expressed as a ratio of the trace quantity of β-actin band of the same sample. It was calculated using "Quantity one" computer program, version 4.6.3.
a :Krausakul Wallis Test ;b :Chi Square test;**p < 0.01: is highly significant.

Table 6: Semi quantitative RT-PCR for measurement of MCM5 Positivity Rate in the Malignant Group Compared to Benign and Normal Control Groups.

Group	*Semi quantitative FOXM1 by RT PCRa	No. of cases › .99(%)[b]
Normal control:		
Median	0.002	1/20(5%)
Range	-.0424_.2454	
Mean Ranks	23.05	
Benign:		
Median	0.002	2/17(11.8%)
Range	.0664_.6640	
Mean Ranks	30.15	
Malignant:		
Median	1.3008	
Range	.8972_1.4960	26/37 (70.3%)
Mean Ranks	48.69	
X^2	25.063	30.173
P	0.000**	0.000**

*Normalized qtyis the trace quantity of the FOXM1 band (average intensity x mm2)expressed as a ratio of the trace quantity of β-actin band of the same sample. It was calculated using "Quantity one" computer program, version 4.6.3.

a :Krausakul Wallis Test; b :Chi Square test; **p < 0.01: is highly significant.

Table 7: Semi quantitative RT-PCR for measurement of FOXM1 Positivity Rates in the Malignant Group Compared to Benign and Normal Control Groups.

Parameter	Sensitivity	Specificity	PPV	NPV	Accuracy
Qualitative MCM5	78.30%	75.60%	76.30%	77.70%	75.60%
MCM5 by Semiquantitation	78.30%	81%	80.50%	78.90%	79.70%
Qualitative FOXM1	70.20%	83.70%	81.20%	73.80%	77%
FOXM1 by Semiquantitation	70.20%	91.89%	89.60%	75.50%	81%
Qualitative MCM5 &FOXM1	94.50%	64.80%	72.90%	92.30%	79.70%
MCM5& FOXM1by Semiquantitation	94.50%	78.30%	81.30%	93.50%	86.40%

Table 8: Performance characteristics of Investigated Markers for Detection of Breast Cancer [14].

Parameter	Sensitivity	Specificity	PPV	NPV	Accuracy
Qualitative MCM5	87.50%	75.60%	60.80%	93.30%	79.20%
MCM5 by Semiquantitation	87.50%	81%	66.60%	93.70%	83%
Qualitative FOXM1	62.50%	83.70%	62.50%	83.70%	77.30%
FOXM1 by Semiquantitation	62.50%	91.80%	76.90%	85%	83%
Qualitative MCM5 &FOXM1	100%	64.80%	55.10%	100%	75.40%
MCM5 & FOXM1 by Semiquantitation	100%	78.30%	66.60%	100%	

Table 9: Performance characteristics of Investigated Markers in early stage (0+1) Breast Cancer [16].

MCM5 and FOXM1 expression in low grade disease (grade 1)

The overall sensitivities of MCM5 and FOXM1mRNA by either qualitative or semi quantitative RT-PCR in Low Grade Breast Cancer were 88.8% and 66.6%. Furthermore the specificity increased after semi quantization of MCM5 and FOXM1mRNA in Low Grade Breast Cancer from 75.6% and 83.7% to 81% and 91.8% respectively.

The combined sensitivity of MCM5 and 1FOXMmRNA by either

qualitative or semi quantitative PCR in Low Grade Breast Cancer was 100%, While their combined specificities were 64.8% and 78.3%, respectively Table 10.

Parameter	Sensitivity	Specificity	PPV	NPV	Accuracy
Qualitative MCM5	88.80%	75.60%	47%	96.50%	78.20%
MCM5 by Semi-quantitation	88.80%	81%	53.30%	96.70%	82.60%
Qualitative FOXM1	66.60%	83.70%	50%	91.10%	80.40%
FOXM1 by Semi-quantitation	66.60%	91.80%	66.6%	91.80%	86.90%
Qualitative MCM5 &FOXM1	100%	64.80%	40.90%	100%	71.70%
MCM5& FOXM1 by Semi-quantitation	100%	78.30%	52.90%	100%	82.60%

Table 10: Performance characteristics of Investigated Markers in Low Grade Breast Cancer.

No significant correlation between MCM5 and FOXM1 expression in breast tissue samples detected by either qualitative or semi quantitative RT-PCR and any of clinicopathological factors including parity, family history, Body Mass Index (BMI) previous hormonal therapy and pathological type.

Discussion

Worldwide, breast cancer is the most common cancer among women. Meanwhile it is most curable when detected at its earlier stages. According to the American Cancer Society, there are currently more than 2.8 million breast cancer survivors in the United States [21]. The field of biomarker discovery has been recently the subject of intense research and activity. Early detection and treatment of cancer in its pre-invasive state is expected to greatly aid cancer control efforts [22]. The development of novel biomarkers would definitely help achieve this goal.

So there is an urgent need to retrieve promising breast cancer biomarkers with adequate sensitivity and specificity. Criteria for identification and prioritization of marker candidatesneed to take into account both clinical relevance and technical feasibility [23].

MCM5 was chosen form highly correlated breast cancer genes from breast cancer database and FOXM1 was chosen form genes highly correlated to MCM5 retrieved from public breast cancer databases, thus this study aimed at assessing the pathogenic value and clinicopathological correlates of MCM5and FOXM1 in breast cancer.

MCM5 is a part of MCM5 complex protein. The MCM2-7 proteins are present in all phases of the proliferative cell cycle but are absent in the quiescent, terminally differentiated and senescent out of cycle states. Since most of human cells are in out of cycle states therefore MCM5used as potential biomarker to detect maturation of cells and malignancy [7]. MCM5mRNA was detected in colorectal cancer, and cervical cancer [24,25].

FOXM1 was found to be differentially expressed in most solid tumors [26]. Moreover, it is implicated in the carcinogenesis of more than 20 types of human tumors [27]. FOXM1 is widely expressed in breast epithelial cell lines and is increased in transformed breast epithelial cell lines. Consistently, FOXM1 expression is elevated in breast carcinomas [28].

To the best of our knowledge, this study is considered the first to detect the correlated expression of MCM5 and FOXM1 in breast tissue samples of breast cancer patients by conventional qualitative RT-PCR and semi-quantitative RT-PCR.

In the current study, MCM5 was reported as a novel potential biomarker to detect breast cancer cases. The results revealed that the positivity rate of conventional RT PCR for breast tissue samples MCM5mRNA level in malignant group was (76.3%) as compared to benign group (21.1%) and (2.6%)of the normal breast tissue samples($p<0.01$).The overall sensitivity, specificity, PPV, NPV and accuracy of this method was (78.3%, 75.6%, 76.3% ,77.7% and 75.6%)respective. Recent study identified MCM5mRNA aberrant expression in breast cancer tissue consistent with the mRNA-10b target regulation (29%).

As regards FOXM1, the positivity rate of conventional RT PCR for breast tissue samples FOXM1mRNA level in malignant group was (81.3%), as compared to benign group (15.6%)and (3.1%)of the normal breast tissue samples ($p<0.01$). The overall sensitivity, specificity, PPV, NPV and accuracy of this method was (70.2%, 83.7%, 81.2%, 73.8% and 77%) respectively. These results go hand in hand with those reported by Kretschmer et al. [31] who reported that FOXM1 was the most significantly over expressed gene in breast cancer by Microarray and quantitative RT-PCR among seven up regulated genes in breast cancer including FOXM1. FOXM1 was 140 fold increased in Invasive Ductal Carcinoma (IDC) tissuesand 100 fold increased Ductal Carcinoma in situ (DCIS) tissues compared to normal breast tissues, using immune histochemistry.

Bektas and colleagues [31] analyzed FOXM1 expression in

invasive breast cancer and normal breast tissues on a tissue microarray. They found a strong cytoplasmic expression of the transcription factor FOXM1, resulting most likely from it's strong over expression. Additionally, using RT-PCR, FOXM1 was found to be over expressed in breast cancer compared to normal breast tissue on both the MRNA and protein level.

Francis and coworkers [32] examined the differences in FOXM1 mMRNA levels between non-tumor and tumor tissues and they found a significant; three fold rise ($p<0.001$) of FOXM1 mMRNA level in tumor tissues, indicating that over expression of FOXM1 has a potential role in breast cancer tumor genesis. They also showed that there were no significant variations in FOXM1 mMRNA level between grade 1/2 and 3 patients ($p=0.271$)($p<0.01$ and <0.01, respectively).

Discrepancies in breast tissue FOXM1mRNA sensitivity in different reports could be explained by the different types of samples used, in which the number of living cells varies, proper transport and storage and other factors. In the present study, trials were done to limit these factors as much as possible. Preservation of breast tissue samples at -80^{0}C and finally using housekeeping gene, ß actin, to exclude samples with degraded mRNA

To provide further insight into the role of MCM5 and FOXM1 in early detection of breast cancer, we compared qualitative RT PCR with semi quantitative RT PCR. In the latter technique, values were presented as a ratio of the specified gene's signal divided by that of the β-actin signal in the same sample.

The median levels of MCM5, and FOXM1 were increased to 1.64, 1.30 respectively in the malignant group as compared to the benign group 0.712, 0.002 respectively, and 0.002, respectively in the healthy normal group.

We determined the threshold value for optimal sensitivity and specificity of MCM5 and FOXM1mRNA by semi quantitative RT PCR using (ROC) curve i.e. Receiver operating characteristics curve (Figure 2, 3) which was formed by calculating the true positive fraction (sensitivity percent) and false positive fraction (100-specificity) at several cut off points [33]. Accordingly, the best cut off value (by considering the benign and healthy normal groups as a control group) for MCM5 and FOXM1mRNA were (0.865 and 0.99 respectively)(area under the curve were 0.865 and 0.99 respectively). Applying this cut off value, the overall sensitivity and specificity of MCM5 and FOXM1mRNA were (78.3%,70.2%) and(81%,91.8%), respectively.

The combined sensitivity of MCM5 and 1FOXMmRNA by either qualitative or semiquantitative among the different groups was 94.5%, but the specificity increased after semiquantitative PCR from 64.8% to 78.3%, respectively.

This study included 16 early stage (0+1) Breast Cancer cases. Their overall sensitivities of MCM5 and FOXM1mRNA by either qualitative or semi quantitativeRT-PCR were 87.5% and 62.5% respectively. Furthermore, the specificity of MCM5, FOXM1mRNA in early stage increased after semi-quantization from 75.6% and 83.7% to 81% and 91.8% respectively. The combined sensitivity of MCM5 and FOXM1mRNA by either qualitative or semiquantitativein early stage (0+1) Breast Cancer was 100%, While their combined specificities were 64.8% and 78.3%, respectively.

This study included 9 Low Grade Breast Cancer cases and their overall sensitivities of MCM5 and FOXM1mRNA by either qualitative or semiquantitative RT-PCR were 88.8% and 66.6%. Furthermore, the specificity of MCM5 and FOXM1mRNA in Low Grade Breast Cancer increased after semi quantization from 75.6% and 83.7% to 81% and 91.8% respectively.

The combined sensitivity of MCM5 and 1FOXMmRNA by either qualitative or semiquantitativePCR in Low Grade Breast Cancer was 100%, While their combined specificities were 64.8% and 78.3%, respectively.

So, the combination allows a better sensitivity for the detection of breast cancer at pre-invasive/early stage, invasive breast cancer which suggests the potential usefulness of this combination for early diagnosis. Moreover, our data gives an additional insight about the potential role played by these aberrations in the carcinogenic pathway of early breast cancer.

Conclusions

MCM5 and FOXM1are two novel biomarkers that may be exploited to improve breast cancer early detection as well as therapeutic targeting. Further studies are warranted in these directions.

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No finding has been received for this study.

Informed consent

All relevant blood samples were taken from the patients after appropriate informed consent.

Research involving Human Participants and/or Animals

Appropriate ethical approval was taken for the study from the ethics committee of Ain shams university hospitals.

Disclosure of potential conflicts of interest

The authors declare no conflicts of interest

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