

Clinical significance of β -catenin, hTERT, p53, and Wnt7A as biomarkers for ovarian cancer

E. Khatoon^{1,2}, N. Deka¹, M. Deka², K.K. Saikia², M.N. Baruah³, G.N. Ahmed³

¹Department of Obstetrics and Gynaecology, Gauhati Medical College, Assam

²Department of Bioengineering & Technology, Institute of Science and Technology, Gauhati University, Assam

³North East Cancer Hospital and Research Institute, Jorabat, Guwahati, Assam (India)

Summary

Aim: The aim of the present study was to examine the association of β -catenin, hTERT, p53, and Wnt7A with the clinicopathologic features of epithelial ovarian carcinoma (EOC). **Materials and Methods:** By using qRT-PCR method, the authors attempted to elucidate the diagnostic evaluation of β -catenin, hTERT, p53, and Wnt7A mRNA for ovarian malignancy. **Results:** It was observed that, compared to the healthy control group, the expression levels of β -catenin, hTERT, p53, and Wnt7A were upregulated in all the ovarian cancer cases. The current study indicated that individual expression of β -catenin, hTERT and Wnt7A did not significantly correlate with patients' clinicopathological parameters. However, expression of p53 significantly correlated with the FIGO Stage ($p < 0.001$) and histological grade ($p < 0.001$) of EOC patients. **Conclusions:** The mRNA expression levels of β -catenin, hTERT, p53, and Wnt7A were upregulated in all the ovarian cancer cases, compared to the healthy controls, which signifies their roles as ovarian cancer biomarkers. These biomarkers can be recognized as a potential important target for detection and anticancer therapies for ovarian cancer cases.

Key words: Wnt7A; p53; β -catenin; hTERT; Clinicopathological.

Introduction

Ovarian cancer is the fifth leading overall cause of death from cancer in women and the most common cause of gynaecologic mortality according to WHO cancer statistics, 2015. Approximately 75% of women with ovarian carcinoma present with Stage III or IV disease as early diagnosis is frequently difficult because of vague abdominal symptoms at presentation (abdominal pain, bloating, gastrointestinal or urinary tract complaints).

In India, the North East (NE) region is seeing a marked increase in cancer incidence and deaths. The NE tribes and the genome pool of the region are quite distinct from the rest of India. The potential risk factors may be due to the diversity in genome pool, food habits, genetic susceptibility or role of some other factor associated with it. The age adjusted incidence rate (AAR) of ovarian cancer in NE India ranged from 1.5 to 10.7 per one Lakh populations according to the National Cancer Registry Programme, 2013.

The World Health Organization (WHO) classifies ovarian neoplasms according to their histological differentiation, namely epithelial, sex cord-stromal, and germ cell neoplasms [1]. Epithelial ovarian cancer (EOC) represent the largest group and are basically subdivided into serous, mucinous, endometrioid, clear cell, and transitional cell tumors [1].

β -catenin, encoded by β -catenin gene (CTNNB1), is a

component of the Wnt signalling pathway and plays a well-defined role in cellular adhesion [2]. Wnts are secreted glycoproteins that bind to the members of the seven transmembrane family of proteins called Frizzleds (Fzds) [3]. The levels of β -catenin are regulated by a protein complex that promotes its degradation by the ubiquitin–proteasome pathway via glycogen synthase kinase-3 β (GSK-3 β) mediated phosphorylation of amino-terminal β -catenin sequences [4, 5]. When the Wnt pathway is activated, degradation of β -catenin is inhibited, resulting in accumulation of β -catenin, and transcriptional activation of target genes such as c-myc, cyclin D, matrix metalloproteinase-7 (MMP-7), and cyclooxygenase-2 [5].

The present study focused on WNT7A as it is exclusively expressed in epithelial cells in the female reproductive tract [6]. Furthermore, WNT7A increases cell proliferation via activation of the canonical WNT/ β -catenin pathway [7]. Mutation and deregulation of the components of Wnt/ β -catenin pathway in many human cancers are known to be the cause for tumorigenesis [4].

Human telomerase reverse transcriptase (hTERT) is an important factor during tumourigenesis and its level is elevated during an early stage of tumourigenesis [8]. In most normal human somatic cells telomerase is repressed, and without new synthesis of telomeres, with progressive cell division the chromosomes shorten, eventually triggering

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apoptosis [9]. In contrast, cancer cells escape from replication limitations due to reactivation of telomerase, which is believed to be an essential step during malignant tumour progression [10]. Thus, the telomere length can be stabilized at almost any length after activation of telomerase, apparently without influencing the viability of the tumor cells [11].

p53 is one of the most commonly mutated tumor suppressor genes and it is altered in 50% of advanced cases of ovarian cancer [12]. Stimuli like UV irradiation-induced DNA damage, inappropriate proto-oncogene activation, mitogenic signaling, and hypoxia have been demonstrated to activate p53 [13]. In normal cells the level of wild-type p53 is low or is in undetectable steady state, but when mutated within its core DNA binding domain, p53's normal instability is abrogated which leads to oncogenic gain-of-function along with massive accumulation of steady state mutant p53 protein [14]. In response to cellular stress p53 induces apoptosis and inhibition of the p53 pathway accelerates cancer progression and development of resistance to chemotherapy [15, 16]. However, there is a paucity of data on aspects regarding association of β -catenin, hTERT, p53, and Wnt7A mRNA expression with clinicopathological characteristics of patients with ovarian carcinoma from North-east India. The purpose of this study is to determine the clinical significance of β -catenin, hTERT, p53, and Wnt7A mRNA as tumor markers and their association with the clinicopathologic features of EOC.

Materials and Methods

Patients greater than or equal to 12 years of age with histologically proven ovarian cancer were included in the study, while those having a history of malignancy other than ovarian cancer were excluded, as that could affect compliance with the protocol or interpretation of results. Forty-six tissue samples were collected over the period July, 2016 to May, 2018 from Department of Obstetrics & Gynaecology, Gauhati Medical College, Assam and North East Cancer Hospital & Research Institute, Jorabat, Assam. The population consisted of patients diagnosed with ovarian cancer of serous, mucinous, endometrioid, and unclassified type of carcinoma. Clinical follow up data were available for all patients until death or June, 2018. The mean age of the patients was 48.7 (range 17-85) years. Available data included the patients' age, tumor FIGO (International Federation of Gynecology and Obstetrics) Stage, tumor histology, and tumor grade (Table 1).

Informed consent was obtained from each patient included in the study. Approval for the study was provided by the Institutional Ethics committee of Gauhati Medical College and Hospital (No. MC/217/2016/76) and North East Cancer Hospital & Research Institute (R. No. ECR/776/Inst/As/2015). Samples were collected following proper ethical guidelines and all the samples were properly stored at -80°C until the time of analysis.

Total RNA was extracted from the surgically removed tumor using RNASure Mini Kit (Cat no. NP-84105). Those with 260/280nm absorption ratio > 1.8 were immediately subjected to cDNA synthesis. Complementary DNA was synthesized using 1 μ g of RNA and 1 μ l of random primer (200 ng/ μ l) to a total reaction volume of 12 μ l. The contents were incubated in a thermal cycler

at 65°C for five minutes. Reverse transcription was carried out by adding the contents to the reverse transcription mixture consisting of 4 μ l of 5X RT buffer, 1 μ l RNAase inhibitor (40 U/ μ l), 2 μ l 10 mM dNTPs mixture, 1 μ l Moloney murine leukemia virus reverse transcriptase (M-MuLV RT, 200U/ μ l). For reverse transcription, the thermal cycling profile was: 25°C for ten minutes, 42°C for one hour, and 70°C for ten minutes. The cDNA thus formed was stored at -20°C after proper labeling till further use.

Quantitative PCR (qPCR) was performed in StepOnePlus Real-time PCR System. Previously published primers were used for β -actin [17], β -catenin [18], p53 [19], WNT7A [20], and for hTERT specific primer sequences were designed using NCBI primer blast (Table 2). Each reaction mixture contained: 5 μ l Power SYBR Green PCR Master Mix, 0.25 μ l each of 10 pmol forward and reverse primers, 1 μ l cDNA, and 3.5 μ l nuclease free water. Cycling conditions were as follows: activation of the AmpliTaq Gold Polymerase at 95°C for ten minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, and annealing and extension at 60°C for one minute, with a melting curve analysis performed at the conclusion of the PCR assay.

Real-time quantitative RT-PCR was performed for β -catenin, hTERT, p53, and Wnt7A genes with β -actin as a reference gene for the normalization of the target gene expression levels and with the normal ovary tissue as control sample. The normalized relative β -catenin, hTERT, p53 and Wnt7A expression level in tumor tissues vs. the control sample was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) [21]. All data are presented as mean \pm standard deviation (SD) values. Data were analyzed using SPSS version 20 software package. The differences in β -catenin, hTERT, p53, and Wnt7A mRNA expression with different clinicopathological parameters of EOC patients were evaluated by the Mann-Whitney and Kruskal-Wallis non-parametric test. The error bars on the graphs represented the SD. The *p* values of less than 0.05 were considered significant throughout (95% confidence interval).

Results

The mean age of the patients was 48.7 (range 17-85) years, and nearly half ($n = 30$, 65.2%) were diagnosed with advanced stage EOC (FIGO Stage III and IV). The most common histology was serous type ($n=20$, 43.5%). Majority of the patients were diagnosed with grade 2/3 EOC ($n=40$, 86.9). Detailed characteristics of the patients are described in Table 1.

The differential gene expression level for β -catenin, hTERT, p53, and Wnt7A were determined by means of qPCR. Relative quantification (RQ) values of β -catenin, hTERT, p53, and Wnt7A were normalized to the endogenous control β -actin and were expressed as $2^{-\Delta\Delta Ct}$ (fold change) relative to control. The amplification plot as well as the melting curve analysis of each gene are presented in Figure 1 (a and b). The melting curve analysis performed after the completion of PCR confirmed the Tm (melting temperature) of amplified template. The amplification from the specific product of β -actin, β -catenin, hTERT, p53, and Wnt7A were displayed with a Tm of 84.2°C, 77.1°C, 74.7°C, 80.5°C, and 87°C, respectively. Mean \pm SD values

Table 1. — Characteristics of the epithelial ovarian cancer patients.

Clinical features		Number of patients (%)
Mean age (year, range)		48.7 (17-85)
FIGO Stage	Early (I, II)	16 (34.8%)
	Advanced (III, IV)	30 (65.2%)
Histology	Serous adenocarcinoma	20 (43.5%)
	Malignant ovarian neoplasm (unclassified)	13 (28.3%)
	Mucinous adenocarcinoma	9 (19.6%)
	Endometrioid adenocarcinoma	4 (8.6%)
Histologic Grade	1	6 (13%)
	2 or 3	40 (87%)

Table 2. — Genes analyzed and primer sets used.

Genes	Primer sequence (5'-3')	Amplicon size	Reference
β -actin	ACGTGGACATCCGAAAGAC	308 bp	[17]
	CAAGAAAGGGTGTAAACGCAACTA		
β -catenin	AAAATGGCAGTGCCTTAG	100 bp	[18]
	TTTGAAGGCAGTCGTCGTA		
p53	TAACAGTCCCTGCATGGCGGC	121 bp	[19]
	AGGACAGGCACAAACACGCACC		
hTERT	AAACCTTCCTCAGCTATGCC	276 bp	Designed using NCBI primer blast
	TGACGCGCAGGAAAAATGTG		
WNT7A	AGTACAACGAGGCCGTTCAC	326 bp	[20]
	GCACGTGTTGCACTTGACAT		

of relative gene expression of β -catenin, hTERT, p53, and Wnt7A assessed using qPCR in ovarian cancer samples are presented in Table 3. The RQ ($2^{-\Delta\Delta CT}$) is the fold change compared to the healthy control samples. The control group i.e., the calibrator had a RQ value of 1.

It was observed that, compared to the healthy control group, the expression levels of β -catenin, hTERT, p53, and Wnt7A were upregulated in all the ovarian cancer cases. Significant differences in gene expression between different FIGO stages, histological types, and grades were analysed with Mann-Whitney/Kruskal-Wallis non-parametric test using SPSS 20 software. The *p* values of less than 0.05 were considered significant throughout (95% confidence interval).

The current study indicated that individual expression of β -catenin, hTERT, and Wnt7A did not significantly correlate with patients' clinicopathological parameters. However, expression of p53 significantly correlated with the FIGO stage and histological grade of EOC patients (Table 3, Figure 2).

Discussion

The high mortality in ovarian cancer, the most deadly gynecologic malignancy, is mainly due to late diagnosis. Only less than 20% of ovarian cancer patients are diagnosed at an early stage (FIGO Stage I and II) and the five-year survival rate of these patients is over 90%. However, patients in an advanced stage (FIGO Stage III and IV) have a five-year survival rate of less than 30% as therapies become increasingly ineffective in treating metastatic ovarian cancer [22]. Even after primary cytoreductive surgery and standard first-line chemotherapy, around 70% of ovarian cancer patients relapse with chemoresistance which results in treatment failure and causes over 90% of deaths [23].

India has the second highest mortality rate for ovarian cancer after China and North east (NE) India is often called as India's 'cancer capital' due to its highest cancer incidence rate in the country. The reason for this can be attributed to a combination of genetic factors, lifestyle choices, dietary habits, low awareness, and late detection. According to a study in NE population by Sharma *et al.*, the age-standardized rates (ASR) for ovarian cancer was recorded to be 9.8 per 100,000 females, with 7.8% of total cancer cases in females [24]. EOC is the most common ovarian malignancy. This heterogeneous disease has several histologic subtypes that show characteristic cytogenetic features, oncologic signaling pathways, molecular signatures, and clinical-biologic behavior.

Based on pathogenetic mechanisms, recent findings suggest a dualistic model of ovarian carcinogenesis that consists of types I and II. Type I (low-grade serous, mucinous, and endometrioid) cancers manifest as large adnexal masses with early-stage disease with an overall good prognosis. They commonly arise from well-described, genetically stable precursor lesions (usually b-

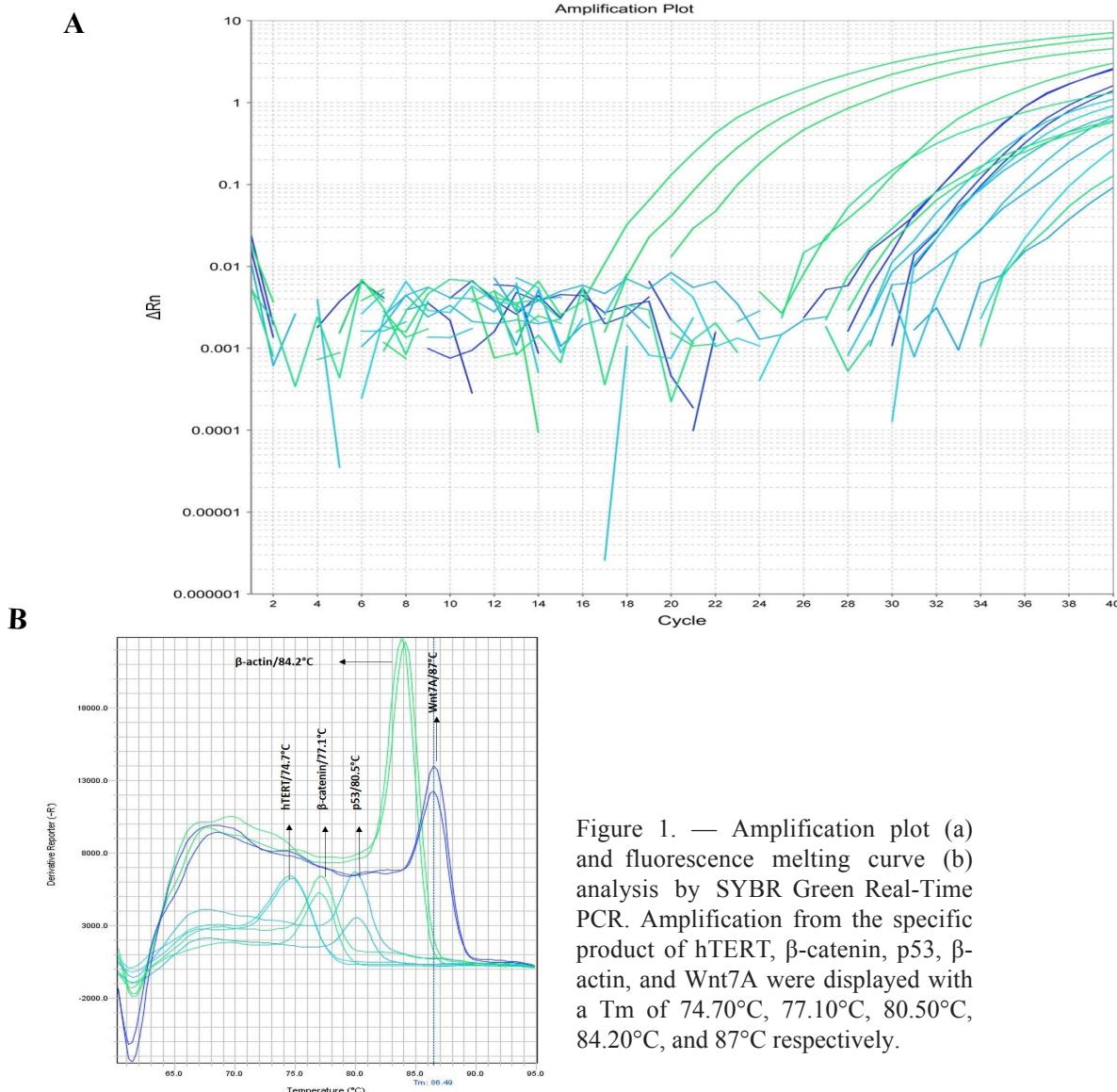


Figure 1. — Amplification plot (a) and fluorescence melting curve (b) analysis by SYBR Green Real-Time PCR. Amplification from the specific product of hTERT, β -catenin, p53, β -actin, and Wnt7A were displayed with a T_m of 74.70°C, 77.10°C, 80.50°C, 84.20°C, and 87°C respectively.

orderline tumors) and are low-grade serous, mucinous, and endometrioid cancers. In contrast, type II carcinomas originate de novo from the adnexal epithelia, have aggressive biologic behaviour and often demonstrate chromosomal instability. Type II carcinomas are generally high-grade serous, endometrioid, mixed, and undifferentiated variants [25].

The key event in the transduction of the Wnt signal is likely the stabilization of β -catenin. In the absence of Wnt signal, β -catenin is targeted for degradation in the proteasome. In the presence of Wnt ligands, the Frizzled receptor is activated, leading to the repression of glycogen synthase kinase-3 β (GSK3 β) and consequently the accumulation of β -catenin and its translocation to the nucleus [26]. There, β -catenin forms a complex with the nuclear transcriptional regulator T-cell factor/lymphoid enhancer factor (TCF/LEF) to promote the expression of Wnt target genes [26].

It was observed that, compared to the healthy control

group, the expression levels of β -catenin and Wnt7A were upregulated in all the ovarian cancer cases. The present results are in agreement with previous studies demonstrating nuclear β -catenin expression in 16–86% ovary endometrioid carcinomas [27–31]. Zhang *et al.* reported Wnt7a expression was higher in ovarian carcinomas compared with normal ovaries and benign tumors [32]. Upregulation of Wnt7a expression in malignant ovarian carcinomas and its wide expression in highly metastatic epithelial ovarian cancer cell lines signifies that WNT7A can contribute to generation and formation of ovarian cancer in human patients [33]. However, the individual expression of β -catenin and Wnt7A did not significantly correlate with patients' clinicopathological parameters.

The wild-type p53 protein has a very short half-life and is detected in low levels, whereas the mutant p53 protein has increased stability and it accumulates predominantly in the nucleus of neoplastic cells [34]. In the present study,

Table 3. — Comparison of tumour marker expression according to clinicopathological parameters in epithelial ovarian cancer patients.

		β -catenin expression		hTERT expression		p53 expression		Wnt7a expression	
		Fold change	p-value	Fold change	p-value	Fold change	p-value	Fold change	p-value
FIGO Stage	Early (I, II)	9.22 \pm 2.50	= 0.273	39.88 \pm 21.21	= 0.809	6.35 \pm 2.69	\leq 0.001	8.37 \pm 2.29	= 0.061
	Advanced (III, IV)	10.08 \pm 2.33		38.45 \pm 17.94		30.26 \pm 14.82		11.79 \pm 6.49	
Histology	Serous	9.3 \pm 2.81	= 0.324	37.46 \pm 19.99	= 0.927	16.46 \pm 14.89	= 0.612	9.57 \pm 5.54	= 0.520
	Mucinous	9.89 \pm 2.71		36.19 \pm 16.91		19.49 \pm 17.63		11.30 \pm 7.49	
	Others	8.77 \pm 2.43		39.49 \pm 19.19		19.53 \pm 15.58		12.16 \pm 6.49	
Histologic Grade	1	8.75 \pm 2.90	= 0.987	35.50 \pm 9.79	= 0.691	5.61 \pm 2.69	\leq 0.001	10.39 \pm 3.60	= 0.787
	2 or 3	9.13 \pm 4.36		33.74 \pm 7.66		28.81 \pm 14.31		12.04 \pm 6.28	

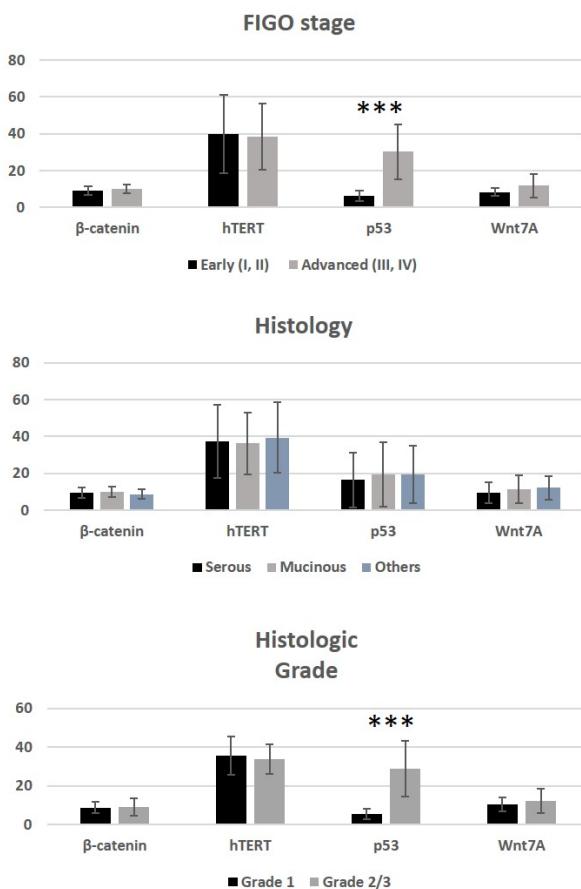


Figure 2. — Bar diagram representing the fold change ($RQ=2^{-\Delta\Delta Ct}$) in mRNA expression of β -catenin, hTERT, p53, and Wnt7A in association with patients' clinicopathological parameters a) FIGO Stage, b) histology, and c) histologic grade. Statistical analysis were performed by SPSS version 20 software package using Mann-Whitney and Kruskal-Wallis non-parametric test.

the upregulated expression of p53 in ovarian cancer cases significantly correlated with the FIGO Stage ($p < 0.001$) and histological grade ($p < 0.001$) of EOC patients. A number of studies have found that p53 mutations are strongly associated with high-grade serous carcinomas, but are rare

in low grade or borderline serous carcinomas [35-38]. O'Neill *et al.* demonstrated a statistically significant higher expression of p53 in high-grade compared with low-grade ovarian serous carcinoma [39]. Lending support to the present study is the observation that p53 is mutated in early-stage high-grade carcinomas [40, 41]. Moreover, Shih Ie and Kurman reported that high- and low-grade serous carcinomas arise via discrete pathways [42]. So, it may be inferred that p53 mutation is required for carcinogenesis and in early event in the pathogenesis of high-grade serous carcinoma.

Telomerase is activated in the majority of human tumors, making it one of the most prevalent biochemical cancer-specific markers [43, 44]. The present findings, in agreement with previous studies, showed upregulation of hTERT mRNA in all the ovarian cancer cases in comparison to the healthy control group. For development of tumors, telomerase activation is crucial and several researchers have demonstrated the telomerase expression in ovarian cancers [45, 46]. Datar *et al.* speculated that hTERT-positive but morphologically non-malignant cells within particular tissues may be susceptible to the subsequent development of invasive cancer. Braunstein *et al.* revealed telomerase activity in ovarian carcinoma cell lines but not in nontrans-formed ovarian cells [47]. Previous studies have demonstrated immunohistochemically that hTERT protein is highly expressed in the ovarian epithelial carcinoma [48, 49]. Thus, measurement of telomerase activity may assist in discriminating malignant from nonmalignant ovarian tumors. Studies of the association between telomerase activity level in cancers and clinical outcome have yielded conflicting results. Previous studies have found that high telomerase activity might be closely correlated with a more aggressive tumor phenotype [46, 50] whereas others have not found such a correlation [51]. The current study indicated no significant correlation between expression of hTERT with patients' clinicopathological parameters.

Conclusion

In conclusion, in the present study the mRNA expression levels of β -catenin, hTERT, p53, and Wnt7A were upregulated in all the ovarian cancer cases, compared to healthy controls, which signifies their roles as ovarian cancer biomarkers. These biomarkers can be recognized as a potentially important target for detection and anticancer therapies for ovarian cancer cases. However, association of these tumor markers as potential prognostic factor was not studied due to short time duration as subjects enrolled at the end of the follow-up period had less time in the study. The current study indicated that expression of p53 significantly correlate with FIGO Stage and histological grade of EOC patients. Thus, the overexpression of p53 most likely represent advanced (III, IV) FIGO Stage and histologic grade 2 or 3 in ovarian carcinoma. Further clinical application of these gene expression as a prognostic and predictive biomarker in EOC should be investigated with larger, and prospective trials.

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Corresponding Author:

E. KHATOON, PhD.

Department of Obstetrics and Gynaecology

Gauhati Medical College

Department of Bioengineering & Technology

Institute of Science and Technology

Gauhati University

781014 Assam (India)

e-mail: elinakhatoon@gmail.com