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Original Article

EGFR, *BRCA1*, *BRCA2* and *TP53* genetic profile in Moroccan triple negative breast cancer cases

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Abstract: Triple negative breast cancer account for 10% to 20% of all newly diagnosed breast cancer cases, this subtype is well known for its lack of estrogen, progesterone and HER2 expression unlike the other subtypes of breast cancer that usually express at least one of the three. The absence of a specific biomarker for TNBC has made his treatment very challenging and his death rates very high compared to the other subtypes. Therefore, in morocco, many studies have been conducted in the hope of finding a specific biomarker for TNBC, but none of these studies has analyzed the EGFR protein expression and its gene molecular profile and correlated the EGFR analyses results with the genetic profile of other genes. In this study, we analyzed EGFR protein expression and the molecular profile of *EGFR, BRCA1, BRCA2* and *TP53* genes in 47 TNBC patients. We conducted a retrospective study of 47 Moroccan patients diagnosed with triple negative breast cancer between early 2013 and 2016. In this study, we have analyzed the EGFR. Protein expression, for all the 47 TNBC patients using pharmDx Kit. Then we used the Ion Personal Genome Machine (PGM) and Ion Ampliseq BRCA1/2 panel and hotspot Cancer panel to analyze the molecular profile of *BRCA1/2* genes and the hotspot regions of *TP53* and *EGFR* genes. The statistical analysis was performed using IBM SPSS Statistics ver. From the 47 analyzed patients using EGFR pharmDx Kit only 16 (34%) had EGFR overexpression while 31 (66%), patients were normal, moreover, From the 47 TNBC patients, only 39 underwent Mutational analysis of *EGFR*, *BRCA1/2*, and *TP53* genes. One patient harbored a *BRCA1* mutation c.798_799delTT (p.Ser267Lys). While for *TP53* gene, 16 patients out of 39 (41%) presented hotspot mutations, seven of them harbored c.743G>A (p.Arg248Gln) mutation, six patients harbored exon 6 mutations from which five harbored the mutation c.659A>G (p.Tyr220Cys) and one the mutation c.817C>T (p.Arg273Cys), and finally, three patients harbored the mutation c.524G>A (p.Arg175His). Regarding *BRCA2* and *EGFR* sequencing results, no mutations or other genetic alterations were detected in 39 patients that were successfully sequenced. Statistical analysis revealed the absence of any correlations.

Keywords: Triple negative breast cancer, *EGFR*, *BRCA1, BRCA2*, *TP53*

Introduction

Triple negative breast cancer is an aggressive subtype of breast cancer that accounts for 10 to 20% of all breast cancer cases, this subtype is well defined by its lack of estrogen (ER), progesterone (PR) and human epidermal growth factor receptor 2 (HER2) expression [1, 2]. In other subtypes of breast cancer, these three biomarkers are essential targets for treatment planes, hence their absence in TNBC makes it treatment very challenging [3]. Therefore, TNBC is usually managed with traditional cytotoxic chemotherapy, which is the mainstay treatment for this subtype. Still, traditional chemotherapy is always associated with high rates of local and systemic relapse [4].

From here, it's clear that TNBC remains without targeted treatment strategies, which increase its aggressiveness and death rats, and emphasize the urgent need for more studies, to better understand the biology of this subtype and discover biomarkers that could be targeted for treatments planes [5]. This fact has constrained the initiation of a molecular classification based

on protein expression, mRNA signatures, and genomic alterations, for TNBC subtype alone [6, 7].

Most of the studies that initiated a molecular classification of TNBC based on protein expression revealed, that EGFR protein is one of the overexpressed proteins in TNBC cells [8], this protein is a surface cell receptor, his activation initiates many other signaling pathways that control cell proliferation, migration, and survival [9, 10]. Unfortunately, half of triple negative breast cancer cases overexpress this protein [11]. EGFR overexpression induces uncontrolled cell proliferation and survival, which contribute to the induction of an aggressive TNBC tumor [12]. To determine the reason behind this EGFR overexpression, genetic screening of the EGFR gene was performed in different studies worldwide. In most of these studies around the world, *EGFR* gene screening shows no gene mutations even though a protein overexpression is well observed. However, two Asian studies that focused on *EGFR* gene in TNBC cases have found an important number of mutations in this gene [13-16]. Other studies that focused on *EGFR* gene amplification, which is another mechanism of EGFR overexpression, has found that up to 24% of triple-negative breast cancer cases harbor EGFR copy number amplification [17-21].

These findings do not explain the high percentage of this protein overexpression in TNBC cases. This indicates the implication of other molecular mechanisms; more scientific studies have proven the implication of other genes. Among these genes, we find in the lead *BRCA1*, *BRCA2*, and *TP53*, punctual mutations [22-24].

Accordingly, we conducted a study in which we analyze EGFR protein expression, and the molecular profile of *EGFR*, *TP53*, *BRCA1* and *BRCA2* genes in TNBC Moroccan patients using next generation technologies. We chose to analyze *TP53*, *BRCA1* and *BRCA2* genes considering the fact that these three are suspected to play a role in EGFR overexpression. Correlating their mutational status with EGFR expression profile could help in determining whether they are implicated or not in this overexpression.

Moreover, In Morocco many studies have been conducted concerning TNBC molecular profile however, there are no studies that analyzed the EGFR protein expression and it gene molecular profile, and correlated the EGFR protein analyzing results with the genetic profile of other genes. Besides, no Moroccan studies have analyzed the *BRCA1/2* genes profile in sporadic TNBC cases and almost all the studies that analyzed *TP53* gene in TNBC used limited analysis technologies such as Sanger sequencing or real time PCR, resulting thereupon on a limited analysis of *TP53* molecular profile in the Moroccan population. In summary, this study will help in determining the frequency of EGFR protein overexpression, plus the molecular profile of EGFR, BRCA1, BRCA2 and TP53 genes in Moroccan TNBC, and analyzing the correlations between these genes and EGFR protein expression profile.

Materiel and methods

Subjects

The current study involved 47 TNBC patients diagnosed at the University Hospital Center in Casablanca and Marrakech, Morocco, between early 2013 and 2016. The primary inclusion criterion was an adequate fresh tumor obtained from a resected tumor sample. The enrolled patients met the following criterion: (I) Pathologically proved TNBC. (II) Complete clinical, pathological and follow-up data. Informed consent was obtained from all recruited patients in this study permitting the use of their surgical specimens and clinic-pathological data for research purposes.

This retrospective study was performed at Anoual Laboratory of Radio-Immuno Analysis, Casablanca, Morocco, where the samples were processed for NGS.

EGFR protein expression

Epidermal growth factor receptor (EGFR) immunostaining was performed on 4 µm FFPE thick sections using EGFR PharmDx Kit (Dako) (2-18C9 anti-bodies) and following the manufacturer's guidelines. The immunohistochemie (IHC) was performed on an automated Benchmark® platform (Ventana Medical Systems) using the UltraView™ universal DAB detection kit (Ventana Medical Systems). The results of EGFR staining was analyzed as follows: 0, no staining or faint membranous staining in <10% of tumor cells; 1+, weak membranous staining in ≥ 10% of tumor cells; 2+, moderate membra-

nous staining in ≥ 10% of tumor cells; or 3+, strong membranous staining in ≥ 10% of tumor cells.

DNA extraction

Genomic DNA was extracted using Qiagen DNA FFPE Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's guidelines. DNA concentration was measured using the Qubit dsDNA HS (High Sensitivity) Assay kit and the Qubit® Fluorometer (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Next generation sequencing using Ion AmpliSeq™ cancer hotspot panel

Ten Nanograms of Genomic DNA was used for the library preparation, multiplex PCR was performed using Ion AmpliSeq Library Kit 2.0 (Thermo Fisher Scientific), Ion Ampliseq Hot Spot panel with one pool for EGFR and TP53 sequencing and Ion Ampliseq BRCA1/2 panel with three pools for BRCA1/2 sequencing. PCR amplicons were partially digested with FuPa reagent. Amplicon product was ligated to barcodes adaptors with IonXpress Barcode kit (Thermo Fisher Scientific). Adaptor ligated libraries were purified using Agencourt AMPure XP reagents (Beckman Coulter, Brea, CA). The library concentration was determined using an Ion Library Quantitation Kit (Thermo Fisher Scientific). Emulsion PCR was carried out using the Ion OneTouch™ System and Ion PGM™ Hi-Q™ OT2 Kit (Thermo Fisher Scientific) according to the manufacturer's instructions fallowed by enrichment step using. Finally Next generation sequencing was performed on the Ion PGM system using Ion PGM Hi-Q Sequencing Kit (Thermo Fisher Scientific).

Data analysis

Generated raw data sequences quality assessment and alignment to the hg19 human reference genome was performed using Torrent Suite software (version 5.10; Thermo Fisher Scientific). Single nucleotide variant calling and couvrage analysis were performed with the Torrent Variant Caller plug-in (version 5.10; Thermo Fisher Scientific), using the recommended variant caller parameter for the ampliseq Hot Spot V2 panel.

Generated variant caller files were used for the annotation of single nucleotide variants, insertions, deletions in the Ion Reporter Server System (Thermo Fisher Scientific) and Ingenuity Variant Analysis software. Only single nucleotide variants with a coverage superior to 50x were consider authentic.

Variants were classified as pathogenic when found reported in Clinvar.

Statistical analysis

Statistical analysis of clinic-pathologic data was performed using IBM SPSS Statistics ver. 21 (IBM Co., Armonk, NY). Chi-square (χ^2) test and Fisher's exact test were performed to assess significance of the association between variables (TP53 and EGFR in TNBC patients) and clinical characteristics. Statistical difference was defined as $P<0.05$.

Results

All the clinical pathological data of the 47 patients included in the study are summarized in **Table 1** plus the expression percentage of ki67 proliferation index. All patients were females with a median age of 46 year old ranging from 34 to 65 year old. Most patients had left sided breast tumor, invasive ductal carcinoma and high histological grade (grade III).

All the 47 patients in our cohort underwent EGFR protein expression. However, Due to DNA extraction failure only 39 Of the 47 patient, underwent targeted DNA sequencing. The clinical-pathological data of the patients according to EGFR expression and to mutational status of *BRCA1*, *BRCA2*, *TP53* and *EGFR* genes are also reported in **Table 1**.

Immunohistochemistry analysis of EGFR protein expression

From the 47 analyzed patients using EGFR pharmDx Kit, 16 (34%) had EGFR overexpression while 31 (66%) patients were normal. The Results of IHC were as follows: 0 in 31 (66%), 2+ in 11 (23.4%), and 3+ in 5 (10.6%), no patient presented a 1+ value for EGFR expression. Correlations between EGFR protein expression and the clinical pathological data of the patients revealed no correlations.

Mutational analysis of EGFR, BRCA1, BRCA2, and TP53 genes

A total of 39 patients in our cohort that were successfully sequenced, for EGFR, BRCA1, BRCA2 and TP53 genes.

Table 1. Clinic-pathologic and biologic data of TNBC patients according to EGFR expression status and mutational status of TP53 and BRCA1

Tumor characteristics		Total (47)	Negative EGFR		Positive EGFR		P	TP53 wild type (23)		TP53 mutated (16)		P	BRCA1 wild type (38)		BRCA1 mutated (1)		P
		N	N	%	N	%		N	%	N	%		N	%	N	%	
Age Range	<40	9	6	12.7	3	6.4	0.382	5	12.8	4	10.2	0.413	8	20.5	1	2.5	0.136
	>40	38	25	53.2	13	27.7		18	46.1	12	30.7		30	76.9	0	0	
Localization	Left	37	25	53.1	12	25.5	0.622	16	41	14	35.9	0.590	29	74.4	1	2.5	0.466
	Right	10	6	12.7	4	8.5		7	18	2	5.1		9	23.1	0	0	
Histopathological type	IDC	39	26	55.3	13	27.6	0.436	19	48.7	14	35.9	0.386	32	84.6	1	2.5	0.560
	ILC	8	5	10.6	3	6.3		4	10.2	2	5.1		6	15.4	0	0	
SBR grade	II	14	10	21.3	4	8.5	0.613	9	23.7	3	7.7	0.818	12	30.8	0	0	0.387
	III	33	21	44.6	12	25.6		14	35.9	13	33.3		26	66.6	1	2.5	
Tumor size	<2 cm	12	9	19.1	3	6.4	0.531	7	18	3	7.7	0.582	10	25.6	0	0	0.438
	>2 cm	35	22	46.8	13	27.7		16	41	13	33.3		28	71.8	1	2.5	
KI-67	20-50%	10	4	8.5	6	12.7	0.365	5	12.8	4	10.2	0.499	9	23.1	0	0	0.466
	>50%	37	27	57.4	10	21.2		18	46.2	12	30.7		29	74.4	1	2.5	

Analyzing EGFR and BRCA2 sequencing results, revealed the absence of mutations in both genes for 39 TNBC patients in our cohort.

BRCA1 sequencing results revealed the presence of one BRCA1 mutation c.798_799delTT (p.Ser267Lys) located in exon 10, no other genetic alterations were reported in all the other sequenced patients. Moreover, no correlations were reported between the clinical pathological data of the patients and BRCA1 mutational status.

Regarding *TP53* gene hotspot sequencing results, mutations were detected in 16 patients out of 39 (41%). Among these 16 TNBC patients; seven patients harbored c.743G>A (p.Arg248Gln) mutation located in exon 7, six patient harbored exon 6 mutations from which five harbored the mutation c.659A>G (p.Tyr220Cys) and one the mutation c.817C>T (p.Arg273Cys), and finally three patients harbored the mutation c.524G>A (p.Arg175His) located in exon 5. Same as BRCA1 and EGFR protein expression no correlations are found between TP53 mutational status and clinical pathological data of the patients.

Correlation between EGFR protein expression and TP53 and BRCA1 genes mutations

From the 39 successfully sequenced patients' only one harbored BRCA1 mutation and an EGFR protein overexpression with a score of +2.

Apropos the correlation between TP53 sequencing results and EGFR protein expression results, only one of our patientS harbored TP53 mutation c.524G>A (p.Arg175His) with an EGFR overexpression value of +2.

From these results, we can predict the absence of a correlation between BRCA1 and TP53 mutations and EGFR overexpression.

Discussion

Although in the last decade, the oncology domain has encountered a huge progression in patient's prognosis, due to the advancement of diagnosis technologies and the appearance of targeted therapies. Unfortunately, due to the absence of molecular markers that allow the use of targeted therapies [4], traditional chemotherapy remains the routine treatment for TNBC. This fact has pushed many scientific researchers to work on finding molecular markers for TNBC, in order to improve patients' prognosis and open the possibility of developing targeted therapies. To this aim, we conducted a study that focuses on analyzing EGFR protein expression and its gene molecular profile in Moroccan TNBC patients.

Knowing that EGFR expression profile contradicts with it gene mutational status in most of the populations worldwide and that EGFR gene has never been analyzed in our population before. This study will allow us to determine this gene profile in the Moroccan TNBC and study the correlation between it mutational status and the protein expression. Moreover, considering that a number of molecular studies suggested the implication of other molecular alterations in EGFR overexpression, Such as BRCA1, BRCA2 and TP53 mutations [22-24]. We decided to analyze the molecular profile of these three genes, and study the correlation between their mutational profile and EGFR protein expression.

In our study results, we report an EGFR protein overexpression in 16 from 47 (34%) TNBC patient, these results are similar to those reported by Kim and al [25]. but less than those reported by Sood and Singh Nigam (86.11%) [26], this difference could be explained by the geographic variations and the difference in the used IHC kits.

The correlation between EGFR over expression and the clinical pathological characteristics of the patients revealed the absence of any association (*P*-value > 0.05) between EGFR overexpression and patient age, histopathological type or grade of the tumors, our results are accordant with other studies [21].

According to our *EGFR* gene sequencing results, no mutations were found in all the sequenced patients, even those with the EGFR overexpression. Other studies worldwide reported similar results [27-29]. No *EGFR* gene mutations were found even though an EGFR overexpression was well observed. Nevertheless, a couple of Asian studies found EGFR mutations in TNBC patients with 11.4% to 21% rate [15, 16]. This difference in *EGFR* genetic profile in TNBC patients between the Asian population and other populations could be explained by

the ethnic and cultural differences. However, the genetic profile of EGFR gene and its protein expression profile are contradicted in most of the world populations and in ours as well. These results persuade us to believe that the presence of a genetic alteration in other genes that regulate EGFR expressions such as *TP53*, *BRCA1* and *BRCA2* [17-19], Maybe the reason behind this EGFR overexpression.

Accordingly, we have decided to analyze the molecular profile of the three genes *BRCA1*, *BRCA2*, and *TP53*, and correlate their mutational profile with EGFR protein expression in hope of highlighting the genetic alterations that correlate with EGFR overexpression. Our sequencing results revealed the presence of 4 different *TP53* mutations in 16 TNBC patients from 39 which represent 41%, similar values were reported by Somali et al. (44%) [3] and Kim et al. (44.3%) [30], but on the other hand higher values were reported by both Dumay et al. (88%) [32] and Weisman et al. (74%) [33]. This value difference could be explained by the fact that both Dumay et al. and Weisman et al. performed a massive sequencing of TP53 gene while in our study we only sequenced the hotspot regions of the *TP53* gene.

In our cohort, seven patients (43,7%) harbored the mutation c.743G>A (p.Arg248Gln) located in exon 7, five patients (31,2%) harbored the mutation c.659A>G (p.Tyr220Cys) located in exon 6, another patient presented the mutation c.817C>T (p.Arg273Cys) located also in exon 6 and the last three patients presented the mutation c.524G>A (p.Arg175His) located in exon 7. The four mutations affect the DNA binding region of the protein, suppressing subsequently their cognition between the protein and his targeted DNA regions. Each mutation functions differently than the other, the two mutations Arg248Gln and Arg273Cys affect the contact between the DNA and the TP53 protein without causing any changes in the tridimensional structure of the protein, they are named (contact mutants) and they are both considered as the most commonly changed residues in breast cancer and TNBC in general. While the two mutations Arg175His and Tyr220Cys change the tridimensional structure of the protein and are named (structural mutants) [34].

According to Immunohistochemistry studies, these mutations always cause TP53 protein overexpression. But, the produced protein will be unable to reenter the nucleus of the cell and stay stuck in the cytoplasm, incapable of fulfilling his duties and functions as a tumor suppress protein[35]. Our results are consistent with other studies that report that most *TP53* mutations in triple negative breast cancer are located in the DNA binding region of the protein and the mutations Arg175His, Arg248Gln, Arg273Cys, and Tyr220Cys are the most common mutations of *TP53* gene in TNBC [35-37].

Apropos the correlation between *TP53* sequencing results and EGFR protein expression results, only one of our patient showed both EGFR overexpression and TP53 mutation, this patient harbored the mutation c.524G>A (p.Arg175His) with an EGFR overexpression value of +2. Many studies have proven that alterations in *TP53* gene could be the cause of the EGFR expression instability, thus altered *TP53* can active a recycling pathway of EGFR protein which causes the presence of a high concentration of EGFR protein due to the over recycling in comparison to the degradation [38]. No study has analyzed the effect of this particular *TP53* mutation on EGFR protein overexpression.

Regarding the sequencing results of *BRCA1* gene, only one patient presented a *BRCA1* mutation (2.5%) in all the 39 successfully sequenced patients, in our study all the 39 TNBC patient were selected randomly, with no family history for cancer, therefore these results will be compared to the results of similar studies in which *BRCA1* was sequenced in sporadic cases of TNBC. The prevalence of *BRCA1* mutations in our population was very low compared to other studies that sequenced *BRCA1* in sporadic TNBC [39-42].

Our patient harbored the c.798_799delTT (p.Ser267Lys) mutation, it's a frameshift mutation located in exon 10, and causes the deletion of two thymine nucleotides, creating thereupon a stopping codon in position 285 of the protein, and affecting the NLS domain of the protein. This mutation has been reported previously by Tazzite et al. [43] in the Moroccan population then by Laraqui et al. [44] in a north African population, and it has been reported as well in otherpopulations around the world [45-48].

The *BRCA1* mutation in our TNBC patient co-occurred with an EGFR protein overexpression as well, with a score of +2. The coexistence of this *BRCA1* mutation (p.Ser267Lys) with EGFR overexpression, theorize the possibility of a relationship between both alterations. Abramovitch et al. [49] and Maor et al. [50] has proven that *BRCA1* interacts with Sp1 specify protein 1 and prevents his association with the IGF-IR receptor. That plays the role of a transcription factor for some of EGFR promoters. Subsequently, an altered *BRCA1* will be enabled to block the interaction between Sp1 and IGF-IR, this latter will stay active causing, therefore, an over activation of EGFR expression.

These findings don't prove or disprove the implication of TP53, BRCA1 and BRCA2 in EGFR overexpression and more studies should be conducted in hope of finding the factors behind this overexpression.

Conclusion

In this study, we analyzed EGFR protein expression profile in Moroccan TNBC, and the molecular profile of *EGFR*, *BRCA1*, *BRCA2* and *TP53*, to report their frequency in Moroccan TNBC and study their correlations with EGFR protein expression.

Our results revealed the presence of a high EGFR overexpression and *TP53* gene mutations in Moroccan TNBC, and a very low *BRCA1* mutation frequency. While, for EGFR and *BRCA2* no mutations were reported. Moreover, our study proves that *EGFR* gene mutations does not explain the EGFR protein overexpression in the Moroccan population, and we report the absence of any correlation between *TP53*, *BRCA1* genes and EGFR expression profile.

More studies should be conducted worldwide to investigate the implication of other molecular factures in EGFR protein overexpression.

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Disclosure of conflict of interest

None.

Abbreviations

ER, Estrogen Receptor; FFPE, formalin-fixed paraffin-embedded; HER-2, Human Epidermal Growth Factor Receptor 2; HS, High Sensitivity; mTOR, mechanistic target of rapamycin; OT2, One Touch 2; TP53, Phosphoinositide 3-kinase; EGFR, Epidermal growth factor receptor; PGM, Personal Genome Machine; PR, Progesterone Receptor; TNBC, Triple Negative Breast Cancer; NLS, Nuclear localization single domaine.

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