

Triple Negative Breast Cancers Have a Reduced Expression of DNA Repair Genes

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Abstract

DNA repair is a key determinant in the cellular response to therapy and tumor repair status could play an important role in tailoring patient therapy. Our goal was to evaluate the mRNA of 13 genes involved in different DNA repair pathways (base excision, nucleotide excision, homologous recombination, and Fanconi anemia) in paraffin embedded samples of triple negative breast cancer (TNBC) compared to luminal A breast cancer (LABC). Most of the genes involved in nucleotide excision repair and Fanconi Anemia pathways, and *CHK1* gene were significantly less expressed in TNBC than in LABC. *PARP1* levels were higher in TNBC than in LABC. In univariate analysis high level of *FANCA* correlated with an increased overall survival and event free survival in TNBC; however multivariate analyses using Cox regression did not confirm *FANCA* as independent prognostic factor. These data support the evidence that TNBCs compared to LABCs harbour DNA repair defects.

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Introduction

Triple negative breast cancer (TNBC) defines a clinical subset of breast cancer negative for estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor type 2 (HER2). It accounts for about 15% of breast tumors and is characterized by an aggressive clinical course with therapeutic resistance, high rates of local and systemic relapse, and poor survival. This is probably due to the intrinsic biology of this type of tumor as well as to the absence of specific targeted treatments such as hormonal therapy used for ER positive tumors and trastuzumab/lapatinib for HER2 over-expressing tumors [1,2].

TNBC encompasses more than one subtype [3,4]. Morphologically they include metaplastic, adenoid cystic and medullary like and at a molecular level they display different mRNA profiles such as basal and claudin type [5,6]. Cumulating evidence suggest that the triple negative phenotype on clinical assays enriches for basal-like cancer, but no complete overlap exists between the two groups [7,8]. Indeed, 25% of the TNBCs are not basal-like on gene expression profile and similarly there are basal-like tumors that are not triple negative by immunohistochemistry.

Most of the tumors that develop in women with germline mutations in the *BRCA1* breast cancer susceptibility gene are TNBC [9,10]. *BRCA1* has a crucial role in the repair of double-

strand breaks and its mutation leads to cancer predisposition and genomic instability [11,12]. *BRCA1* related TNBCs share several pathological features with sporadic TNBCs and cluster within the basal subtype by gene expression profile [13]. Thus it is not surprising that there are data suggesting that a proportion of sporadic basal-like breast cancers may have a dysfunctional *BRCA1* pathway, due to gene promoter methylation or transcriptional inactivation [13,14]. Deficits in other DNA-repair pathways, such as base excision repair (BER) inactivation [15], MTMG promoter methylation [16] and lack of hOGG1 [17] have been reported in TNBCs. These tumors also exhibit higher DNA copy alterations and loss of heterozygosity than other breast cancer types, suggesting higher genomic instability [18–20]. These latter data, together with the reported association between *BRCA1* deficiency and TNBC, suggest that DNA repair alterations may be important for the development of this tumor type. If this is the case, there could have important prognostic and predictive implications, such as the response to the currently used anticancer agents and to novel targeted agents, as the poly-ADP-ribose polymerase (PARP) inhibitors [21].

While tests for the evaluation of DNA repair defects in primary cells or in clinical samples have been described [22,23], to our knowledge validated functional assays for the quantification of the tumor DNA repair capabilities to be used in clinical setting do not

exist. There are however some biomarkers that, even if not completely validated [24–27], have been used as surrogate of DNA repair functionality, i.e. the mRNA and/or expression levels of key proteins involved in DNA repair pathways. Aim of our study was to evaluate in a multiparametric manner the mRNA expression level of different key player of DNA repair pathways (i.e. base excision repair-BER-, nucleotide excision repair-NER- and homologous recombination- HR- and Fanconi anemia- FA) in TNBCs compared with LABCs and to correlate with clinicopathological patient characteristics.

Materials and Methods

Patients and samples

150 formalin-fixed paraffin embedded core breast cancer samples, diagnosed as triple negative (80 cases) and luminal A (70 cases), were retrospectively collected from patients who came to the medical observation at the Breast Care Unit, A.O. Istituti Ospitalieri di Cremona, Italy. Ethical permission for the study was obtained from the A.O. Istituti Ospitalieri di Cremona (Italy) ethical committee. Written informed consent was obtained from patients, if applicable. All the samples were anonymized by a pathologist staff member and none of the researcher conducting the gene expression analysis had access to disclosed clinicopathological data. The patients characteristics are summarized in Table 1.

Definition of molecular classes

Tumour grade was evaluated using the modified Bloom and Richardson method. Immunohistochemical evaluation was performed on paraffin-embedded tumour samples obtained at diagnosis. Estrogen receptor, progesterone receptor, HER2 staining were carried out at the Pathology Unit of the A.O. Istituti Ospitalieri di Cremona, Italy. The immunohistochemical method used for routine markers is fully described elsewhere [28]. Tumors were classified as: luminal A (hormone receptor positive: estrogen receptor + and/or progesterone receptor + and HER2-), and triple-negative (ER-ve, PR-ve, HER2-ve) [29].

RNA and real time

RNA was extracted from core biopses with the High Pure FFPE RNA Micro Kit (Roche). RNA concentrations were then measured by NanoDrop spectrophotometer and retro-transcription to cDNA was performed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystem) in a 96well plate, one for each histological subgroup. 15ug are preamplified with TaqMan Preamp Master mix (Applied Biosystem) and with the pool of primers used later for real time PCR reactions. Genes selected have a key role in the BER (*PARP1*); in the NER pathway (*ERCC1*, *XPA*, *XPD*, *XPF* and *XPG*); in the FA pathway (*BRCAl*, *FANCA*, *FANCC*, *FANCD2*, *FANCF* and *PALB2*); in DNA damage checkpoint pathway (*Chk1*). Optimal primer pairs (Table 2) were chosen, spanning splice junctions, using PRIMER-3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and the specificity was verified by detecting single-band amplicons of the PCR products. The EP Motion robot (Eppendorf) dispensed 10 ng of cDNA in triplicate on a 384 wells plate, one for each gene and for each subgroup. The reaction was performed with Sybr Green PCR master mix (Applied Biosystem) and the curve of dissociation was evaluated. A standard curve for each specific gene was included in each plate for an absolute quantification of the copy of RNA. Samples were then normalized using the absolute copy number of the housekeeping gene (cyclophilin A). The two different sample plates (TNBC and LABC) were calibrated using inter-run calibrators samples (four different breast cancer cell lines) to correct for run to run technical variation. The calibration procedure was performed on a gene per gene basis.

Data and statistical analysis

The Mann-Whitney U test was chosen to compare clinicopathological and gene expression data between the two groups of TNBCs and LABCs. The median is taken as valid proxy for expression level of all genes. Relationships between family gene expression levels were examined using the Spearman's rank correlation. Each gene distribution was split into three expression levels (low, intermediate and high level) defined by the two tertiles calculated on patients who did not die at the end of the study. For

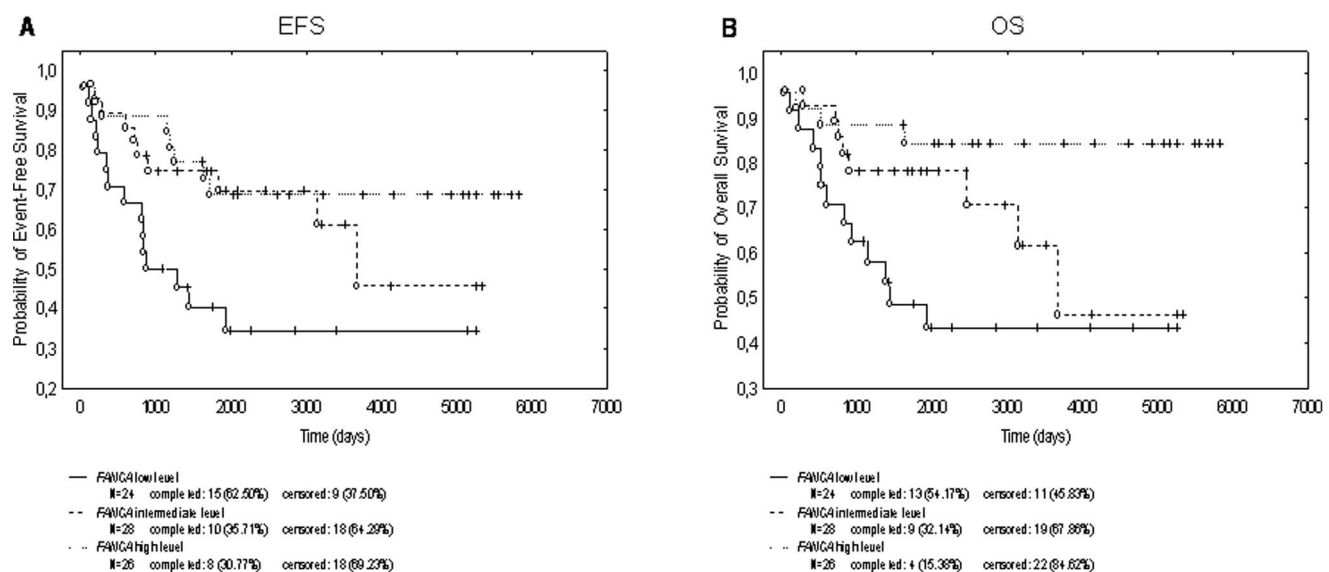


Figure 1. Kaplan-Meier estimates for event-free survival (panel A) and overall survival (panel B) in TNBC patients stratified by the different level of FANCA expression (univariate analysis, categorization based on tertiles).

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Table 1. Baseline patients' demographic.

CLINICAL PARAMETERS	TNBC (N = 80)	LABC (N = 70)
Follow-up time – years		
Median (range)	5.5 (0.1–16.0)	3.16 (0.6–17.3)
Mean ± SD	6.7±4.7	5.9±5.5
Age – years		
Median (range)	59.5 (33.1–91.3)	66.6 (36.5–84.5)
Mean ± SD	60.2±14.4	63.1±11.5
Age – no. (%)		
<65	46 (57.5)	31 (44.3)
≥65	34 (42.5)	39 (55.7)
Menopausal status – no. (%)		
Pre-	19 (23.7)	12 (17.1)
Post-	59 (73.8)	58 (82.9)
not known	2 (2.5)	0 (0.0)
Clinical tumor size – no. (%)		
≤20 mm	16 (20.0)	21 (30.0)
>20 mm	36 (45.0)	12 (17.1)
not known	28 (35.0)	37 (52.9)
Tumor size at surgery – no. (%)		
≤20 mm	36 (45.0)	42 (60.0)
>20 mm	22 (27.5)	11 (15.7)
not known	22 (27.5)	17 (24.3)
Pathological T staging – no. (%)		
T1	41 (51.3)	48 (68.6)
T2	20 (25.0)	11 (15.7)
T3	2 (2.5)	1 (1.4)
T4	4 (5.0)	3 (4.3)
not known	13 (16.2)	7 (10.0)
Pathological N staging – no. (%)		
N0	37 (46.3)	66 (94.3)
N+	18 (22.5)	0 (0.0)
not known	25 (31.2)	4 (5.7)
Metastatic status – no. (%)		
M0	60 (75.0)	68 (97.1)
M1	4 (5.0)	0 (0.0)
not known	16 (20.0)	2 (2.9)
Pathological staging – no. (%)		
Stage I	28 (35.0)	48 (68.6)
Stage IIA	13 (16.3)	11 (15.7)
Stage IIB	5 (6.2)	1 (1.4)
Stage IIIA	4 (5.0)	0 (0.0)
Stage IIIB	4 (5.0)	3 (4.3)
Stage IV	4 (5.0)	0 (0.0)
not known	22 (27.5)	7 (10.0)
Basal histotype – no. (%)		
IDC	66 (82.5)	23 (32.9)
ILC	5 (6.2)	16 (22.9)
Other	7 (8.8)	30 (42.8)
not known	2 (2.5)	1 (1.4)
Basal tumor grade – no. (%)		

Table 1. Cont.

CLINICAL PARAMETERS	TNBC (N = 80)	LABC (N = 70)
1	1 (1.2)	6 (8.6)
2	14 (17.5)	47 (67.1)
3	64 (80.1)	15 (21.4)
not known	1 (1.2)	2 (2.9)
Basal Ki67 – no. (%)		
Median (range)	37 (3–90)	7.5 (1–16)
Mean ± SD	44.6±26.4	7.3±3.3
<10	3 (3.7)	50 (71.4)
≥10	75 (93.8)	20 (28.6)
<20	15 (18.7)	70 (100.0)
≥20	63 (78.8)	0 (0.0)
not known	2 (2.5)	0 (0.0)
Neoadjuvant treatment – no. (%)		
Done	35 (43.8)	19 (27.2)
not done	38 (47.5)	50 (71.4)
not known	7 (8.7)	1 (1.4)
Surgical treatment – no. (%)		
Mastectomy	21 (26.3)	14 (20.0)
Breast conservation	48 (60.0)	54 (77.1)
not known	11 (13.7)	2 (2.9)
Vital status – no. (%)		
Alive	53 (66.2)	69 (98.6)
Dead	27 (33.8)	1 (1.4)

SD: standard deviation; IDC: invasive ductal carcinoma; ILC: invasive lobular carcinoma.

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Table 2. Real Time PCR primers.

GENE	PRIMER F	PRIMER R
	CICLOPHILIN A	GACCCAACACAATGGTTCC
	TTTCACCTTTGCCAACCA	
PARP1	AAGAAATGCAGCGAGAGCAT	CCAGTGTGGGACTTTTCCAT
ERCC1	GGCCTATGAGCAGAAACCAG	TTCACGGTGGTCAGACATTC
XPA	GAACCACTTTGATTTGCCAAC	TGTTTTGCCTCTGTTTTGG
XPF	GAGAAATCTTTTTGTGAGGAAACTG	CAACTTCAGTTTGTGCTGTTT
XPG	AAGCCATCAAACCTGCCTTC	TCGTTTTCTCTCGAACTTGG
XPD	GTGGCCATCAGCTCCAAT	CAGCAGGAGGTTCCCATAGT
BRCA1	GCCAGAAAACACCACATCAC	CAGTGTCCGTTACACACAA
FANCA	GAGGTTCTTCAGTCATACCCTGA	TCTCTGTCATGTAACAGCA
FANCC	CCAGCCAGAGTTCTTTGAGG	CGAAGCCAGAGGCAGACTAC
FANCD2	CCCATCTGCTATGATGATGAA	CGTATTTGCTGAGGGGATATG
FANCF	GCTAGTCCACTGGCTTCTGG	GGTGGCGGCTAGTCACTAAA
PALB2	TGGGACCTTTCTGATCAAC	GGGGCATCAAAAATTGGTTT
CHK1	GGTCACAGGAGAGAAGGAAT	TCTCTGACCATCTGGTTACGG

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Table 3. Normalized and calibrated values (mean \pm SD and median) of the different DNA repair genes in tumor samples.

PATHWAY	GENE	TNBC (N=80)		LABC (N=70)		p value*
		Mean \pm SD	Median	Mean \pm SD	Median	
BER	<i>PARP1</i>	10.87 \pm 10.92	8.250	7.05 \pm 5.46	5.221	0.0002
NER	<i>ERCC1</i>	0.71 \pm 0.66	0.633	6.87 \pm 20.77	1.040	<0.0001
	<i>XPA</i>	0.10 \pm 0.08	0.080	0.16 \pm 0.21	0.104	0.0309
	<i>XPF</i>	23.35 \pm 55.61	5.108	68.93 \pm 107.05	21.996	<0.0001
	<i>XPG</i>	0.66 \pm 0.71	0.468	1.51 \pm 3.52	0.287	0.1534
	<i>XPD</i>	0.276 \pm 0.360	0.186	6.68 \pm 10.26	3.662	<0.0001
FA	<i>BRCA1</i>	0.028 \pm 0.025	0.020	0.83 \pm 3.36	0.062	<0.0001
	<i>FANCA</i>	0.138 \pm 0.279	0.057	0.27 \pm 0.71	0.094	0.8293
	<i>FANCC</i>	0.005 \pm 0.007	0.003	0.002 \pm 0.050	0.003	0.3627
	<i>FANCD2</i>	0.08 \pm 0.124	0.054	4.45 \pm 23.32	0.150	<0.0001
	<i>FANCF</i>	0.026 \pm 0.027	0.018	0.451 \pm 1.170	0.031	<0.0001
	<i>PALB2</i>	0.453 \pm 0.563	0.306	5.91 \pm 23.03	2.010	0.0006
SENSOR	<i>CHK1</i>	0.179 \pm 0.300	0.108	11.51 \pm 46.44	1.779	<0.0001

*p value indicate the difference of the gene expression levels between TNBC and LABC; p value in bold are significant.
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further testing, each gene distribution was dichotomized (low and high level) using the median gene expression value as the cut-off point. Consistently, patients were divided into different subgroups based on different gene expression level. Univariate comparisons for all categorical variables were performed by Pearson's chi-squared test with Yates's correction for continuity or Fisher's exact test, when appropriate. Overall survival (OS) and event-free survival (EFS) were classified as outcome measures and defined as the length of time from the start of neoadjuvant chemotherapy or the date of curative surgery (in patients treated with only adjuvant

therapy) or the date of diagnosis (in patients diagnosed with metastases) to the last follow-up date or death (irrespective of the cause) or to the first relapse or progression event. OS and EFS curves were plotted with the Kaplan-Meier method. The log-rank test and Cox proportional hazard models were used to compare time-to-event distributions between subgroups. All analyses were performed using SPSS (version 10.0– Chicago, IL) and STATISTICA software system (version 6– Tulsa, OK). All tests were performed two-sided and p values <0.05 were considered statistically significant.

Results

Patients' characteristics

We considered a cohort of 150 paraffin embedded core biopsies, 80 with a pathological diagnosis of TNBC and 70 with a diagnosis of LABC that came to the medical attention of a single medical center, the Breast Care Unit of A.O. Istituti Ospitalieri di Cremona from January 5th, 1995 to August 19th, 2008. The two groups (TNBC vs LABC), whose characteristics are depicted in Table 1, were comparable in terms of age ($p=0.2056$) and menopausal status at diagnosis ($p=0.4493$), surgical treatment ($p=0.3200$), pathological T staging at surgery ($p=0.1404$), and follow-up time ($p=0.0649$).

mRNA expression of the 13 genes involved in DNA repair pathways

Genes involved in different DNA repair pathways were studied by RT-PCR and Table 3 summarizes the data for both TNBC and LABC. There was considerable variability in expression levels in the individual genes. The levels of the NER genes, *ERCC1*, *XPA*, *XPF* and *XPD* genes were significantly lower in TNBCs than in LABCs, while there was no difference for *XPG*. FA genes were less expressed than NER genes in TNBC. *BRCA1*, *FANCD2*, *FANCF* and *PALB2* genes were significantly less expressed in TNBCs than in LABCs. *CHK1* gene was significantly less expressed in TNBCs than in LABCs. On the contrary, *PARP1* levels were higher in TNBCs than in LABCs.

Table 4. Significant p values for the association between gene expression and clinic-pathological characteristics of triple negative breast cancer patients (univariate analysis, categorization based on tertiles).

PATHWAY	GENE	pT classification	pN classification	Vital status
		at surgery	at surgery	at last follow up
BER	<i>PARP1</i>			
NER	<i>ERCC1</i>			
	<i>XPA</i>			
	<i>XPF</i>		0.0162	
	<i>XPG</i>	0.0437		
	<i>XPD</i>			
FA	<i>BRCA1</i>			
	<i>FANCA</i>	0.0366		0.0144
	<i>FANCC</i>			
	<i>FANCD2</i>			
	<i>FANCF</i>			
	<i>PALB2</i>			
SENSOR	<i>CHK1</i>			

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Table 5. Overall survival and event-free survival in triple negative breast cancer patients by expression of the different genes (univariate analysis, categorization based on tertiles).

PATHWAY	GENE	EXPRESSION	TNBC (N = 80)	
			OS	EFS
			<i>p</i> value	<i>p</i> value
BER	PARP1	II-I tertiles	0.3056	0.2334
		III-I tertiles	0.6140	0.4632
		III-II tertiles	0.1868	0.3955
NER	ERCC1	II-I tertiles	0.9993	0.9450
		III-I tertiles	0.1868	0.3955
		III-II tertiles	0.2035	0.0957
	XPA	II-I tertiles	0.2035	0.0957
		III-I tertiles	0.7071	0.9908
		III-II tertiles	0.7813	0.9566
	XPF	II-I tertiles	0.7813	0.9566
		III-I tertiles	0.1088	0.4704
		III-II tertiles	0.1088	0.4704
	XPG	II-I tertiles	0.0344	0.0674
		III-I tertiles	0.2870	0.2902
		III-II tertiles	0.1010	0.0764
	XPD	II-I tertiles	0.1010	0.0764
		III-I tertiles	0.1403	0.2480
		III-II tertiles	0.1403	0.2480
FA	BRCA1	II-I tertiles	0.4812	0.6162
		III-I tertiles	0.4196	0.5115
		III-II tertiles	0.1088	0.0408
	FANCA	II-I tertiles	0.1088	0.0408
		III-I tertiles	0.0045	0.0141
		III-II tertiles	0.3579	0.5383
	FANCC	II-I tertiles	0.3579	0.5383
		III-I tertiles	0.0850	0.0736
		III-II tertiles	0.0845	0.1352
	FANCD2	II-I tertiles	0.0845	0.1352
		III-I tertiles	0.1554	0.2216
		III-II tertiles	0.7556	0.9254
	FANCF	II-I tertiles	0.7556	0.9254
		III-I tertiles	0.6192	0.7587
		III-II tertiles	0.5170	0.5381
	PALB2	II-I tertiles	0.5170	0.5381
		III-I tertiles	0.2010	0.1617
		III-II tertiles	0.3056	0.2334
	PARP1	II-I tertiles	0.3056	0.2334
		III-I tertiles	0.6140	0.4632
		III-II tertiles	0.6140	0.4632
SENSOR	CHK1	II-I tertiles	0.7740	0.4396
		III-I tertiles	0.2325	0.2601
		III-II tertiles	0.2325	0.2601

The mRNA expression distribution of all the genes was split into three groups, as described in Materials and Methods. *p* value in bold are significant. doi:10.1371/journal.pone.0066243.t005

Association between gene expression and clinic-pathological characteristics

Table 4 reports the *p* values found between the gene expression level based on tertiles (low, intermediate and high level as specified in Materials and Methods) and some clinic-pathological characteristics in TNBC. Lower levels of *XPG* and *FANCA* were associated with higher pathological T classification at surgery (pT2-4 vs pT1); higher level of *XPF* was associated with no lymph node involvement (pN0 vs pN1-3), and higher levels of *FANCA* correlated with vital status. Using the median gene expression value as a cut-off point of categorization (low and high level), only the association of *XPG* and *FANCA* expression levels with pT classification remained statistically significant. No other associations were found among the other clinic-pathological evaluated parameters (age at diagnosis-65 years vs ≥ 65 years-; menopausal status-pre-menopausal vs post-menopausal-; clinical dimension at diagnosis- ≤ 20 mm vs > 20 mm-; basal histotype-ductal infiltrating

carcinoma vs others-; basal grading-G1-2 vs G3-; basal Ki67- $< 14\%$ vs $\geq 14\%$ and $< 20\%$ vs $\geq 20\%$ -; clinical complete response-cCR vs not cCR-; pathological complete response-pCR vs not pCR-). When the same analysis was performed in the LABC cohort of patients, none association could be found.

We correlated the different gene expression levels based on tertiles with OS and EFS. As depicted in Table 5, the intermediate level of *XPG* correlated on univariate analysis with increased OS compared to the low level. Interesting enough, the high level of *FANCA* correlated with an increased OS and EFS compared to the low level. Again, the intermediate level of *FANCA* correlated with an increase in EFS compared to the low level. The Kaplan-Meier curves (Figure 1) shows that higher level of *FANCA* correlated with an increased OS ($p = 0.0153$) and an increased EFS ($p = 0.0207$). Furthermore, using the median gene expression value as a cut-off point of categorization, we again found a trend (not significant) toward better OS ($p = 0.0906$) and EFS ($p = 0.0999$) in patients with high level of *FANCA*. Multivariate analyses using Cox regression, however, showed that the *FANCA* expression level was not an independent prognostic factor in TNBC patients adjusted for the potential confounding factors: age at diagnosis, surgical treatment, pathological T and N staging at surgery. As regards LABCs, OS and EFS data were not yet mature.

Discussion

TNBC represents a subset of breast cancer with well documented poor prognosis, whose real causes are still to be defined even if a number of adverse factors have been advocated, such as the fact that these tumors are at the diagnosis commonly of high nuclear mitotic grade, of larger tumor size and with a more aggressive expression profile (low BCL2 and high p53 and Ki67 expression) [5,30,31]. Several studies have demonstrated significant lower EFS in patients with TNBC compared to patients with non-TNBC; in addition, shorter OS in TNBC patients is widely reported [3,32–34]. Despite the poor prognosis, these tumors are particularly chemosensitive at least in a short-term time frame [35,36]. In fact, patients with TNBC have increased pathologic complete response (pCR) rates compared to non-TNBC patients after treatment with taxanes and anthracycline agents. Wang et al reported a 38% of pCR in TNBC and 14% in non-TNBC patients treated with taxane in combination with anthracycline [37]. Better response rates were also reported after treatment with alkylating agents, including cyclophosphamide, cisplatin, and carboplatin [38].

TNBC have been shown to harbour DNA repair deficiencies, including *BRCA1* dysfunction, due to promoter methylation or deregulation of other genes involved in their transcriptional regulation [9], BER inactivation [15], MTMG promoter hypermethylation [16] and lack of hOGG1 [17]. Given the importance of the cellular DNA repair in determining the cellular response to different anticancer agents [39–43], *a priori* knowledge of the repair status of a given tumor could play an important role in selection of the most appropriate therapy. Attempts to investigate the functionality of a given DNA repair pathway have been undertaken and shown to be feasible. Willers et al reported the successful detection of *BRCA1*, *FANCD2* and *RAD51* foci in seven breast cancer biopsies irradiated *ex vivo*, suggesting the possibility to detect defects within the complex FA/BRCA DNA damage response pathway [44]. Isolation of epithelial cells from breast tumor specimens and application of specific functional DNA repair assay systems led to the detection of specific defects in double strand breaks repair [22], while homologous recombination status could be determined in ovarian cancer samples by

RAD51 foci formation after *in vitro* treatment with a PARP inhibitor [23]. However, all these assays still need to be technically improved (ie. requirement for an automated foci scoring and setting up of tumor specific primary cultures) rendering difficult their wide spread clinical use. An alternative approach is the study of biomarkers that, even if not completely validated [24–27], can be considered surrogate of DNA repair functionality, i.e. the mRNA and/or expression levels of key proteins involved in DNA repair pathways.

The expression profile of a number of genes involved in the different DNA repair pathways was studied in a cohort of TNBC samples as a surrogate markers of DNA repair status and correlated with different clinic-pathological characteristics, including survival. These data were compared to the ones obtained in a group of patients with LABC, that represent the breast cancer subtype with the best prognosis.

The levels of expression of all the genes involved in the NER and FA pathways were significantly lower in TNBC as compared to LABC samples, with the exception of *XPG*, *FANCA* and *FANCD* whose difference did not reach a statistically significant value.

These expression data might in part explain the extremely high sensitivity of TNBC patients to chemotherapy. It has in fact been demonstrated that not only the deficiency, but also low levels of mRNA/protein involved in NER, i.e. *ERCC1* and *XPF* or *FA* genes are associated with increased sensitivity to alkylating agents in different cellular systems [45]. At the same time, the low expression level of these genes might explain the bad prognosis of the tumors, even if they do respond well to chemotherapy. The low DNA repair capacity would in fact increase the genomic instability of the tumor cells enabling them to accumulate much more mutations through which cells would become more aggressive. Sporadic stage I breast cancer have recently been reported to exhibit a significant deficiency of NER capacity relative to epithelial control tissue [46] by both functional unscheduled DNA synthesis and mRNA expression of NER genes. Low expression of 4 proteins – XPF, FANCD2, MLH1 and pMK2 – assessed in FFPE tumor specimens by semiquantitative immunohistochemistry, was associated with shorter recurrence free survival in multivariate analysis [47], corroborating the idea that tumors with reduced DNA repair capacity will have a higher degree of genomic instability and therefore behave more aggressively.

The data on *FANCA* are, on the contrary, counter-intuitive and intriguing. *FANCA* was not differentially expressed between TNBC and LABC, but it was found to positively correlate in univariate analysis with both EFS and OS (higher level correlated with longer EFS and OS). *FANCA* is one of the 13 proteins cooperating in a

common DNA repair pathway (for rev see [48,49]). *FANCA*, higher expression correlated with an improved outcome that could be a consequence of a less malignant phenotype, due to a more stable tumor genome. This resembles what observed for the expression of *ERCC1* in non small cell lung cancer (NSCLC), where it has been shown that higher *ERCC1* levels were correlated with a better prognosis [50,51], but was also higher levels of *ERCC1* were a marker of resistance to a platinum containing regimens [50]. Recently, the experimental finding that *ERCC1* negative NSLC samples display a higher genomic instability corroborate the more unstable and then the worst clinical history of *ERCC1* low/negative tumors [52].

There are few, but contrasting data, on *CHK1* expression in different breast cancer subtypes. *CHK1* is a kinase that has an important role in both the maintenance of genomic instability and in the transduction pathways after DNA damage [53]. Verlinden et al. [54] reported a twofold higher expression (as assessed by RT-PCR) of *CHK1* in grade 3 TNBC than in other grade 3 tumor breast subtype; while no difference *CHK1* protein expression was found in different breast cancer cell lines. These discrepancies can be related to the different methodologies used (RT-PCR vs western blotting; relative vs absolute RT-PCR values), samples size and the pathological classification of the different tumor analyzed. All tumor samples analyzed expressed *PARP1* mRNA, whose levels were higher in TNBC compared to LABC, reinforcing the data that neoplastic tumors express high level of PARP [55,56].

There are a number of issues that limits the present study: i.e. its retrospective nature, the relative small sample size and the fact it analysed the mRNA expression levels of DNA repair protein rather than the quantification of a functional DNA repair status. Nevertheless, our data are the first to show the mRNA expression of multiple DNA repair genes involved in three different DNA repair pathways (BER, NER and FA) in a cohort of TNBC and LABC.

These data support the experimental evidence that TNBC harbour DNA repair defects and this could explain both the extremely chemo-sensitivity of this tumor type to chemotherapy in a short term and the worse outcome probably correlated with the inability to counteract the increased genomic instability for the lack of an efficient DNA damage status.

Author Contributions

Conceived and designed the experiments: GD DG MB AB. Performed the experiments: ER MG DA RB RG SF VZ LB CF. Analyzed the data: MG DA. Wrote the paper: MG GD DG.

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