Somatic EP300-G211S mutations are associated with overall somatic mutational patterns and breast cancer specific survival in triple-negative breast cancer

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Received: 14 August 2018 / Accepted: 17 August 2018

Abstract

Purpose

We have compared the mutational profiles of human breast cancer tumor samples belonging to all major subgroups with special emphasis on triple-negative breast cancer (TNBC). Our major goal was to identify specific mutations that could be potentially used for clinical decision making in TNBC patients.

Patients and methods

Primary tumor specimens from 149 Norwegian breast cancer patients were available. We analyzed the tissue samples for somatic mutations in 44 relevant breast cancer genes by targeted next-generation sequencing. As a second confirmatory technique, we performed pyrosequencing on selected samples.

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Results

We observed a distinct subgroup of TNBC patients, characterized by almost completely lack of pathogenic somatic mutations. A point mutation in the adenoviral E1A binding protein p300 (EP300-G211S) was significantly correlated to this TNBC subgroup. The EP300-G211S mutation was exclusively found in the TNBC patients and its presence reduced the chance for other pathological somatic mutations in typical breast cancer genes investigated in our gene panel by 94.9% ($P < 0.005$). Interestingly, the EP300-G211S mutation also predicted a lower risk for relapses and decreased breast cancer-specific mortality during long-term follow-up of the patients.

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Conclusion

Next-generation sequencing revealed specific mutations in EP300 to be associated with the mutational patterns in typical breast cancer genes and long-term outcome of triple-negative breast cancer patients.

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Keywords

Breast cancer
EP300 mutations
Triple-negative breast cancer
P53
Next-generation sequencing
Pyrosequencing

The contents of this manuscript has partly been published as an abstract and late-breaking poster at the Annual AACR Meeting in Washington DC, April 2, 2017 (Poster/late-breaking abstract no. 027).

Introduction

Triple-negative breast cancer (TNBC) is one of the major BC subtypes established in clinical use and currently known as the subtype with the worst prognosis [1, 2, 3, 4, 5]. All clinically established breast cancer markers, like

the estrogen receptor (ER), the progesterone receptor (PR), and the human epidermal growth factor receptor-2 (HER-2) are negative in these patients and may, consequently, not be used for clinical decision making in TNBC patients. Moreover, patients with TNBC do not belong to a homogeneous group. In fact, it is now well-known that the term triple-negative breast cancer comprises a wide spectrum of different subtypes with distinct characteristics caused by considerable pathologic and molecular heterogeneity [6, 7]. Transcriptomic analysis of TNBC has revealed at least six distinct subtypes of TNBC [8]. These subtypes include two basal-like, one mesenchymal, one immunomodulatory, one mesenchymal stem-like, and one luminal AR subtype. The discovery of the"luminal-AR" subtype especially has caused increased interest and research focusing on the androgen receptor as a potentially important biomarker in clinical TNBC management [9, 10, 11].

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The intrinsic molecular heterogeneity of TNBC is clinically mirrored by subgroups of TNBC patients with early and frequent relapses and metastasis, while other TNBC patients do not relapse at all, even during long-term follow-up. Thus, it is of high clinical importance to identify novel, clinically useful prognostic and predictive markers for the various TNBC subgroups [12, 13, 14, 15, 16, 17, 18].

Next-generation sequencing (NGS) technologies offer the possibility to assess multiple genes for somatic mutations and may elucidate the driver genetic variations involved in BC carcinogenesis and progression [19]. Several pivotal publications, based on massively parallel sequencing, have presented overviews covering genetic events in TNBC [20, 21]. In general, somatic mutations in TP53 have been shown to be present in the majority of TNBC patients while other mutations occur at lower frequencies. Among these, typical genetic events in TNBC are PTEN mutations, mutations or loss of RB1, FGFR2 amplification, and EGFR amplification [6, 20, 21, 22, 23]. In contrast, the typical mutations observed in the majority of luminal-A/B subtypes (PIK3CA mutations, for instance) are considered to be rare events in TNBC.

The present study employed a novel gene panel consisting of 44 pivotal genes in BC biology. Our major goal was to characterize somatic mutations in the cancer-related genes in different breast cancer subtypes with special emphasis on the TNBC subtype.

EP300 is known to encode an adenoviral E1A-binding protein (also known as p300) involved in multiple cellular processes functioning as a transcriptional co-factor and histone acetyltransferase (HAT) [24, 25]. EP300 is highly homologous to the cyclic AMP response-element-binding (CREB) protein (CBP). EP300 and CBP form a protein complex that has been identified at the promoter regions of more than 16,000 human genes [26] reflecting their role as ubiquitous transcription co-factors.

The biological significance of EP300-G211S mutation in TNBC tumors remains unknown but our results, presented in the following chapters, suggest strongly that it can be used as a "molecular signature" dividing the otherwise heterogeneous TNBC tumors in two distinct molecular subtypes.

Materials and methods

Patients and clinical samples

Sample material was comprised of tumor tissues obtained from 149 patients diagnosed with a primary breast cancer at our hospital (Akershus University Hospital, Norway). Patients` characteristics are summarized in Table 1. The study cohort represented all major breast cancer subtypes (controls) but was enriched for triple-negative breast cancer as the main entity of interest (Luminal-A, $n = 17$; Luminal-B, $n = 20$; HER-2 pos., $n = 26$; TNBC, $n = 86$). The median follow-up time (time from surgery to censoring or death) was 2290 days (6.3 years).

Table 1

Patient demographic and clinical characteristics

	No.	**%**
Total study population	149	100
Breast cancer subtypes		
Luminal-A	17	12.1
Luminal-B	20	14.1
HER-2-positive	26	16.8
Triple-negative BC	86	57.0
ER-positive	51	34.2

PGR positive	36	24.2
HER-2-positive	25	16.8
Grade I	3	2.0
Grade II	42	28.2
Grade III	104	69.8
TNM-classification at surgery (all patients)		
T1	63	42.3
T2	76	51.0
T3	6	4.0
T4	0	0
Tx	4	2.7
N0	84	56.4
N1	40	26.8
N2	12	8.0
N3	8	5.4
Nx	5	3.4
M0	149	100
M1 (at surgery)	0	0
Age at surgery	**Mean**	**Range**
Luminal A	57.1	34.9–80.3
Luminal B	52.3	24.8–77.8
HER-2 pos	57.0	34.9–80.3
TNBC	57.5	26.4–93.9
ER estrogen receptor, *PR* progesterone receptor, *HER-2* human		
growth factor receptor 2, *TNBC* triple-negative breast cancer		

Tumor biopsies were collected at surgery and stored in formalin until paraffin embedded. The formalin-fixed and paraffin embedded (FFPE) blocks were examined by a senior breast cancer pathologist (T.S.) prior to sectioning in order to assure at least 20% tumor cells in each sample. In general, tumor

cellularity was between 30 and 50% in the majority of samples. The microtome and accessories were cleaned with DNA SAP (Thermo Fisher Scientific) prior to sectioning to avoid cross-contamination.

DNA extraction

In order to obtain sufficient DNA from each tumor biopsy, a set of four to six 10 μm tissue curls were cut from each block. The tissue curls were collected in individual sealed sterile tubes and further processed to extract genomic DNA. We used the QIAamp DNA FFPE tissue kit (Qiagen) according to the manufacturer´s protocol.

Next-generation sequencing

DNA samples were analyzed by next-generation sequencing (NGS) using the Human Breast Cancer GeneRead DNAseq Targeted Panel V2 (Qiagen). The panel consists of a collection of PCR primers for targeted enrichment of the coding region of 44 genes commonly mutated in breast cancer (Table 2). Target enrichment and library construction were performed according to the GeneReader workflow (Qiagen) and paired end sequencing was performed on a NextSeq 500 sequencer (Illumina) running 2 × 150 bp chemistry Version 2. Bioinformatics analysis of the sequencing data, including alignment to the reference genome hg19 and variant calling, was performed using Qiagen´s Ingenuity Variant analysis tools [27].

Table 2

List of breast cancer associated genes evaluated by Next-Generation Sequencing (NGS) in this study

Abbreviation	Gene name
ACVR1B	Activin A receptor type 1B
AKT1	AKT serine/threonine kinase 1
ATM	ATM serine/threonine kinase
BAP1	BRCA1 associated protein 1
BRCA1	Breast cancer 1, early onset
BRCA2	Breast cancer 2, early onset
CBFB	Core-binding factor beta subunit

CDH1	Cadherin 1
CDKN2A	Cyclin dependent kinase inhibitor 2A
EGFR	Epidermal growth factor receptor
EP300	E1A binding protein p300
ERBB2	erb-b2 receptor tyrosine kinase 2
ERBB3	Erb-b2 receptor tyrosine kinase 3
ESR1	Estrogen receptor 1, Estrogen receptor α
EXOC2	Exocyst complex component 2
EXT2	Exostosin glycosyltransferase 2
FBXO32	F-box protein 32
FGFR1	Fibroblast growth factor receptor 1
FGFR2	Fibroblast growth factor receptor 2
GATA3	GATA binding protein 3
IRAK4	Interleukin 1 receptor associated kinase 4
ITCH	Itchy E3 ubiquitin protein ligase
KMT2C	Lysine methyltransferase 2C
MAP2K4	Mitogen-activated protein kinase kinase 4
MAP3K1	Mitogen-activated protein kinase kinase kinase 1
MDM2	MDM2 proto-oncogene
MUC16	Mucin 16, cell surface associated
MYC	MYC proto-oncogene, bHLH transcription factor
NCOR1	Nuclear receptor corepressor 1
NEK2	NIMA related kinase 2
PBRM1	Polybromo 1
PCGF2	Polycomb group ring finger 2
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha
PIK3R1	Phosphoinositide-3-kinase regulatory subunit 1
PPM1L	Protein phosphatase, Mg2+/Mn2 + dependent 1L
PTEN	Phosphatase and tensin homolog

PTGFR	Prostaglandin F receptor
RB1	RB transcriptional corepressor 1
RET	Ret proto-oncogene
TP53	Tumor protein p53
TRAF5	TNF receptor associated factor 5
WEE1	WEE1 G2 checkpoint kinase
ZBED4	Zinc finger BED-type containing 4

Pyrosequencing for validation of EP300-G211S mutations

In order to confirm the observed EP300-G211S mutations with a second method, we designed a pyrosequencing assay, a quantitative method capable of determining the proportion of the mutated EP300 gene at the G211S position. The primers that were designed for amplification and sequencing of EP300-G211S mutation are shown in Figs. 1 and 2. Amplification of the target gene was performed using the PyroMark PCR kit (Qiagen) and sequencing of the amplified EP300 region of interest was done using a PyroMark-24 instrument (Qiagen).

Fig. 1

Schematic presentation of the EP300 protein illustrating its functional domains with the location of the missense variants identified in this study: The locations of the amino acid substitutions are indicated by asterisk (*). The nonsense mutations resulting in truncated variants of EP300 protein are labeled in red. LXXLL: LXXLL motif (interaction surface of EP300/CBP complex with nuclear receptor); TAZ 1 and 2: transactivation domain; ZZ: ZZ-type zinc-finger domain; CH1, 2, 3: Cysteine-Histidine-rich domains 1, 2 and 3; BD: bromo domain; PHD: plant homeodomain; HAT: histone acetyltransferase domain; NCBD: nuclear coactivator-binding domain

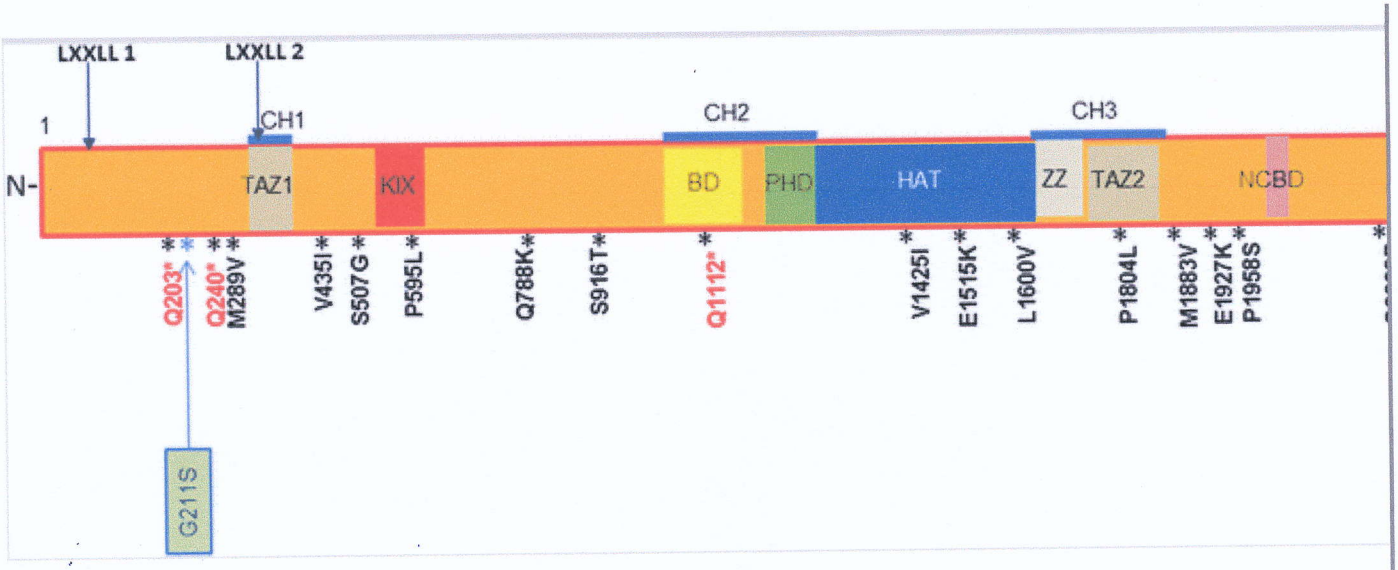

Fig. 2

Location of pyrosequencing primers: The DNA sequences encoding the N-terminal part of EP300 and the amino acid sequence are given. The position of the PCR and sequencing primers are shown by arrows. The position of the G211S mutation is highlighted by a grey background

```
       PCR Forward primer
      >>>>>>>>>>>>>>>>>>>>>>
ggcaatggacaagggataatgcctaatcaagtcatgaacggttcaattggagcaggccga
 G  N  G  Q  G  I  M  P  N  Q  V  M  N  G  S  I  G  A  G  R
            Sequencing primer
            >>>>>>>>>>>>>>>  G211S (GGC>AGC)
Gggcgacagaatatgcagtacccaaacccaggcatgggaagtgctggcaacttactgact
 G  R  Q  N  M  Q  Y  P  N  P  G  M  G  S  A  G  N  L  L  T
gagcctcttcagcagggctctccccagatgggaggacaaacaggattgagaggcccccag
 E  P  L  Q  Q  G  S  P  Q  M  G  G  Q  T  G  L  R  G  P  Q
       PCR reverse primer
    <<<<<<<<<<<<<<<<<<<<<<<
Cctcttaagatgggaatgatgaacaaccccaatccttatggttcaccatatactcagaat
 P  L  K  M  G  M  M  N  N  P  N  P  Y  G  S  P  Y  T  Q  N
```

Location of primers

Forward primer	5'-GGACAAGGGATAATGCCTAATCA-3'
Reverse primer	5'-GGTTGTTCATCATTCCCATCTTAA-3'
Sequencing primer	5'-TGCAGTACCCAAACC-3'

Germline DNA analysis for EP300-G211S variant

A random selection of eleven EDTA-blood samples from EP300-G211S positive TNBC patients were chosen and DNA extracted using Gentra Puregene Blood Kit (Qiagen) to investigate the possibility of the presence of germline G211S

variations. The isolated genomic DNA was analyzed by pyrosequencing as described in the previous section.

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Statistical analysis

A Poisson regression analysis was used to test for an association between EP300G211S mutations and the number of other pathological somatic mutations in typical breast cancer genes. Survival plots were made using the Kaplan–Meier method and the log-rank test was used to test for differences between survival curves. Logistic regression was used to test for the association between EP300G211S mutation and mutations in P53.

Results

We performed next-generation sequencing on a set of 149 biopsy samples obtained from primary tumors of breast cancer patients (Table 1). Due to the given focus on TNBC, the study cohort included a majority of TNBC tumors (n = 86) while control groups consisted of consecutively enrolled luminal-A (n = 17), luminal-B (n = 20), and HER-2 positive (n = 26) subtypes. Actual patient numbers are corrected in figures following exclusion of some (n = 8) samples due to either poor DNA quality or yields. The gene panel used in this study contained 44 breast cancer-relevant genes (Table 2). The barcoded libraries were prepared using 4 different sets of primers covering about 2900 amplicons. The pooled libraries were sequenced from the both ends to achieve an average read depth of 1600. The sequence data were further analyzed by Ingenuity bioinformatics tools [27]. Due to the design of the Ingenuity package, the putative cancer driven mutations are filtered by several criteria including association with clinical data found on COSMIC database, or predicted to be damaging by SIFT [28], SnpEff [29], and PolyPhen-2 [30]. Accordingly, the genetic variants are characterized as pathogenic/likely pathogenic, likely benign, or of uncertain clinical significance due to American College of Medical Genetics and Genomics (ACMG) standards and guidelines [31]. The term likely pathogenic describes a genetic variation/mutation that with a greater certainty than 90% may promote cellular cancer transformation. Using the Ingenuity software package we selected the mutations that were characterized as pathogenic or likely pathogenic and turned out to be a subset of 15 genes (given in Fig. 3a, b). Overall, the highest frequency of mutations was observed in TP53 and PIK3CA, described in further detail below.

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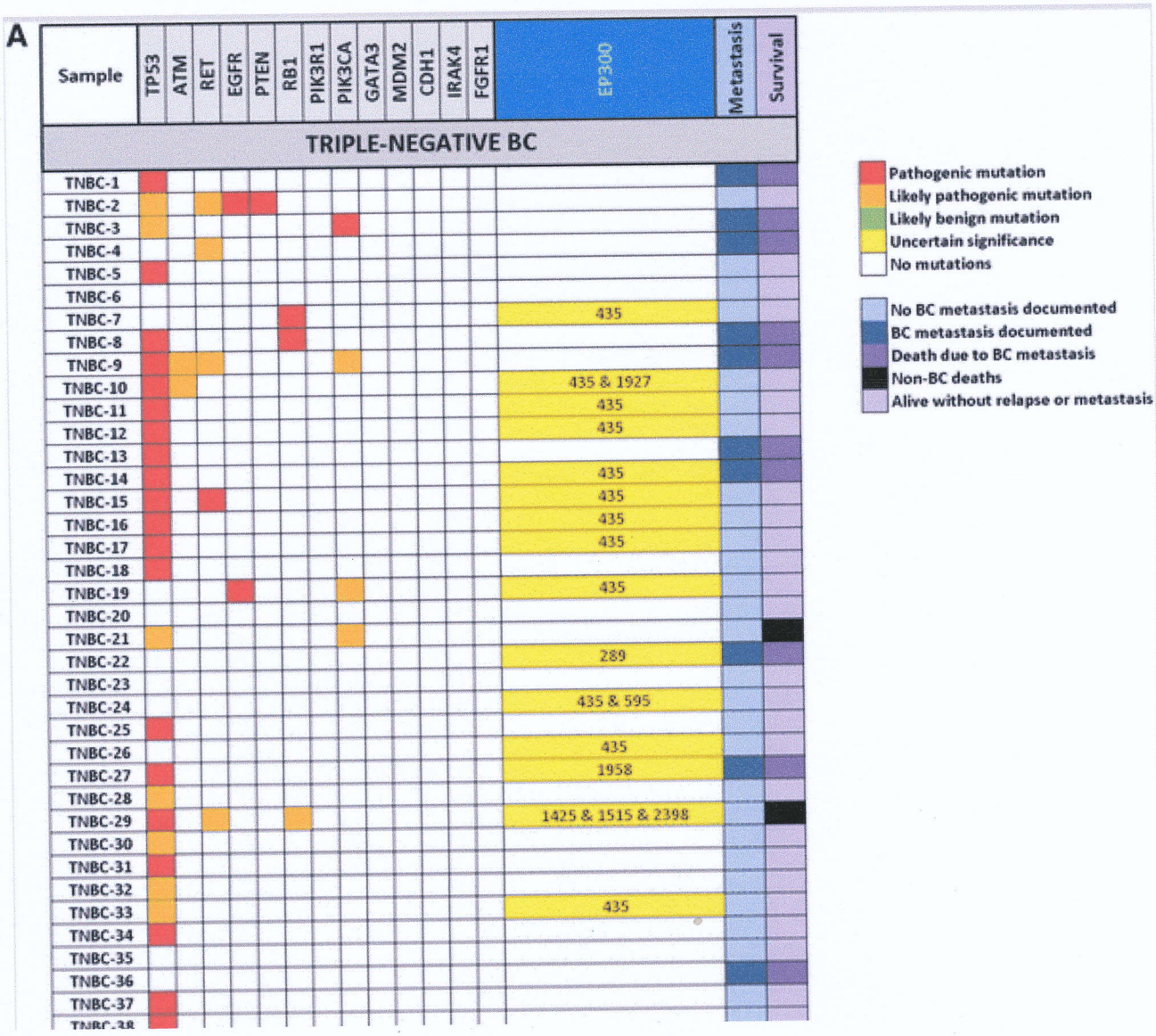
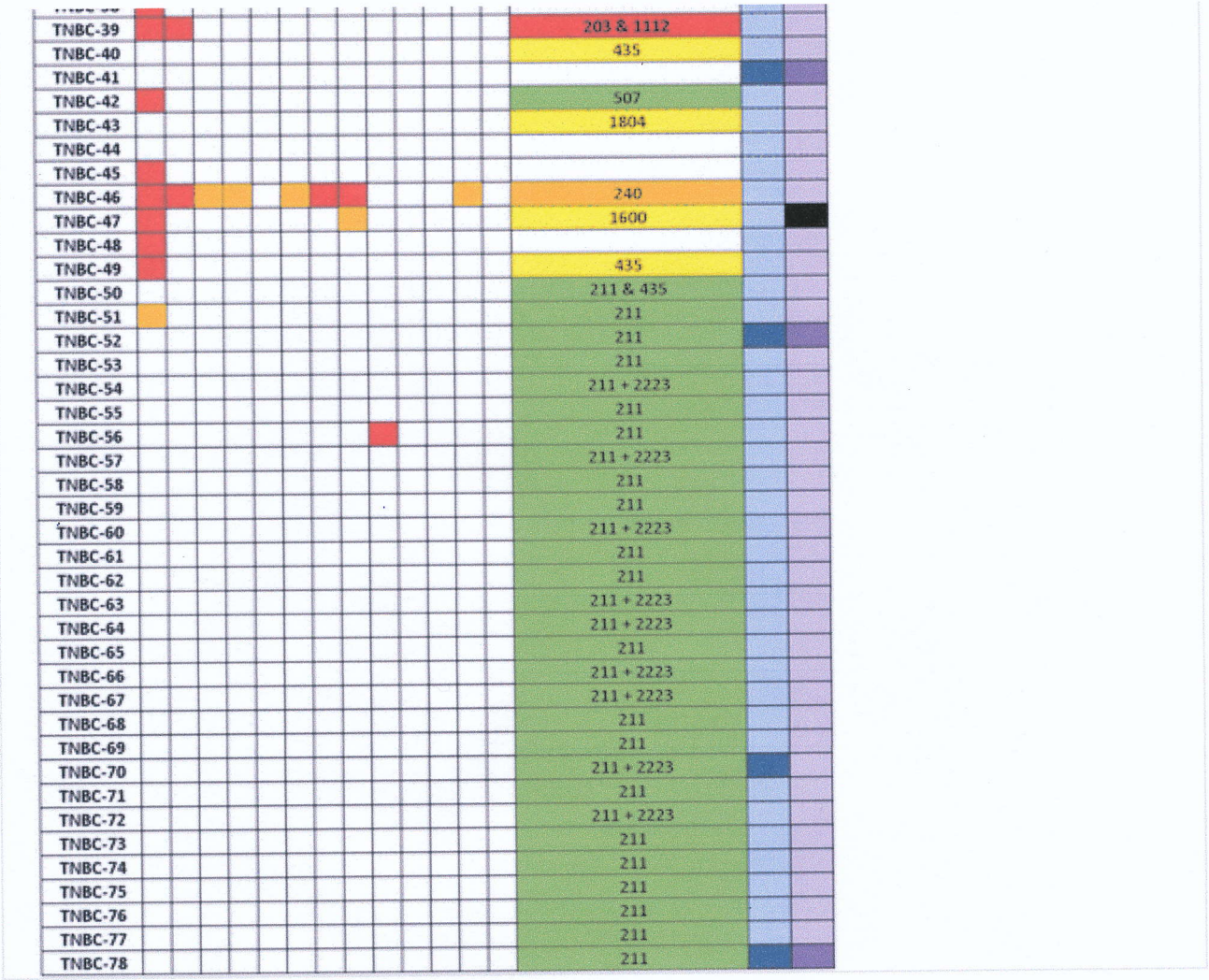
Fig. 3

a, b Pattern of somatic mutations in typical breast cancer genes: **a** TNBC patients; **b** LUM-A, LUM-B and HER-2 pos. patients. The figures give an overview of the genetic variants as detected by Next-Generation-Sequencing (NGS). The variants represented in this panel were selected according to their role in breast cancer pathogenesis and are classified as"pathogenic" or"likely pathogenic" according to the ACMG-classification [31]. Due to their high frequencies, the variants in the gene encoding E1A binding protein p300 (EP300) are represented even they are mostly described as"likely benign" or of"uncertain significance" due to criteria used by Ingenuity analysis software [27]. EP300 variants are depicted in order of amino acid residues: 203(Q203* stop gain); 211(G211S); 240(Q240* stop gain); 289(M289V); 435(V435I); 507(S507G); 595(P595L); 788(Q788K); 916(S916T); 997(I997V); 1112(Q1112* stop gain); 1425(V1425I); 1515(E1515K); 1600(L1600V); 1804(P1804L); 1883(M1883V); 1927(E1927K); 1958(P1958S); 2223(Q2223P); 2398(D2398N)

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240
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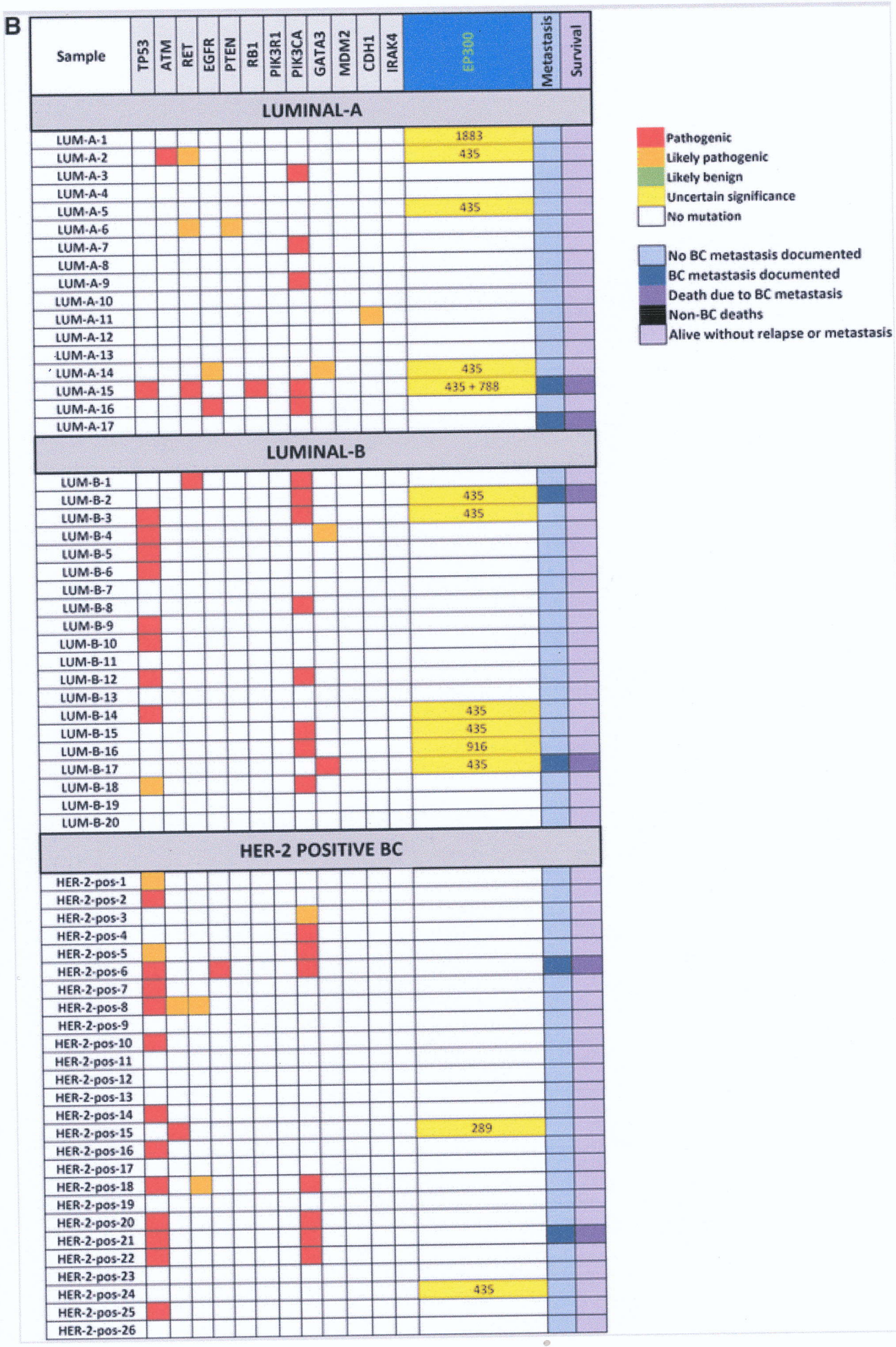
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Sample
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ATM
RET
EGFR
PTEN
RB1
PIK3R1
PIK3CA
GATA3
MDM2
CDH1
IRAK4
EP300
Metastasis
Survival
LUMINAL-A
LUM-A-1
LUM-A-2
LUM-A-3
LUM-A-4
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HER-2 POSITIVE BC
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HER-2-pos-3
HER-2-pos-4
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HER-2-pos-21
HER-2-pos-22
HER-2-pos-23
HER-2-pos-24
HER-2-pos-25
HER-2-pos-26
289
435
Pathogenic
Likely pathogenic
Likely benign
Uncertain significance
No mutation
No BC metastasis documented
BC metastasis documented
Death due to BC metastasis
Non-BC deaths
Alive without relapse or metastasis

Somatic mutations in TP53

We found TP53 somatic mutations to be the most frequent pathogenic mutations when looking at the entire breast cancer study group (Fig. 3a, b). Thus, 59 of 141 patients (41.8%) had TP53 mutations in their primary tumors. These mutations are classified as pathogenic according to their deleterious effect on the structure and function of the TP53 protein. The majority of the TP53 mutations were missense mutations and mostly found on the DNA binding domain. TP53 mutations were observed in 44.3% of all TNBC patients, 45% of the LUM-B patients and 53.8% of the HER-2 positive patients. In contrast, only 1 of 17 LUM-A patients (5.9%) had a pathological TP53 mutation in her primary tumor, revealing a statistically significant difference ($P < 0.05$) between the LUM-A subgroup and the other subgroups.

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Somatic mutations in PIK3CA

The second most frequently mutated gene in the entire population was the PIK3CA-gene with pathogenic mutations in 23 of 141 patients (16.3%). The highest levels of PIK3CA-mutations were observed in the LUM-B subgroup with 30%, followed by LUM-A patients (29.4%), and HER-2 pos. patients (26.9%). In the TNBC subgroup, only 5 of 78 patients (6.4%) had PIK3CA-mutations in their primary breast tumors. The most common mutation found in our study was a His1047Arg substitution located in the kinase domain of PIK3Ca. This mutation is a well-known hotspot found in a variety of cancer types including breast cancer [32, 33].

Somatic mutations in EP300

The gene encoding the E1A binding protein p300 (EP300) showed to have relatively high rates of somatic mutations in the majority of breast cancer patients in our study (46.5%). However, most of these mutations are described as" likely benign" or of "uncertain significance" (Fig. 3a, b). Interestingly, the specific Glycine211Serine substitution (EP300-G211S) was exclusively detected in triple-negative breast cancer samples. It was present in 29 of 78 TNBC patients (37.2%). In 19 TNBC patients it was the only EP300 mutation detected, while it appeared in 10 patients as part of EP300-double-mutations along with Glutamine2223Proline substitution (EP300-Q2223P). The high frequency of G211S variant was striking (29/78 TNBC patients), raising suspicion that it might be a PCR artifact. The NGS data, however, were

generated by sequencing of two different PCR fragments and the mean of the read depth at this position was 1865.In order to confirm the NGS-generated data, we performed pyrosequencing on all samples bearing the EP300-G211S variation. Results showed that these data were reliable (see in Table 3; Fig. 4 for details).

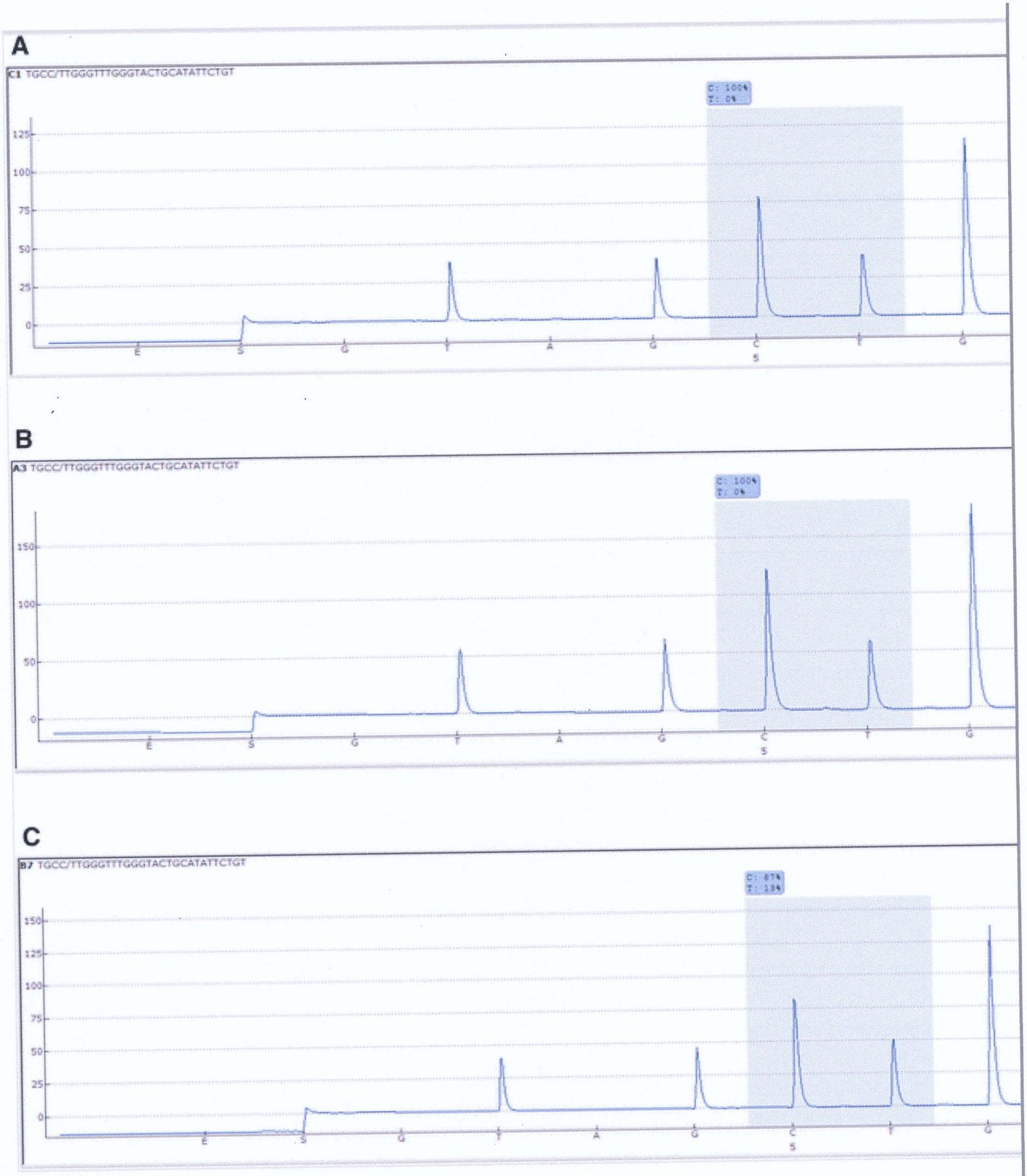
Table 3

Comparison of the pyro sequencing results of the 11 patients chosen to confirm the characterization of the positive G211S variations as somatic along side the VAF from NextSeq NGS

EP300 Pyro sequencing of blood samples G211G/S			
ID	**Pyro sequencing of Blood derived DNA**	**Mean VAF (%) tumor DNA Pyro sequencing**	**VAF (%) tumor DNA NextSeq sequencing**
TNBC-78	WT	15.65	11.4
TNBC-70	WT	9.97	11.4
TNBC-53	WT	11.09	10.3
TNBC-51	WT	13.95	9.2
TNBC-56	WT	11.96	11.2
TNBC-58	WT	16.44	13.4
TNBC-52	WT	8.87	8.8
TNBC-59	WT	19.31	10.6
TNBC-73	WT	13.16	11.5
TNBC-54	WT	13.95	10.8
TNBC-55	WT	13.65	10.8
All values are percent variant allele, or wild type (WT). Pyrosequencing samples were analyzed in duplicate and the VAFs averaged			

Fig. 4

A comparison of three PyroMark Q24 pyrograms of the EP300 codon 211 region. The shaded area of each pyrogram shows the location of the G211S variation found in the TNBC patients; the glycine to serine substitution is caused by a G > A change in genomic DNA, but because of pyromark sequencing primer selection, the region is being sequenced in reverse and the variation thus reveals itself as a C > T nucleotide change on the resulting G211S positive pyrogram. PyroMark Q24 sequencing is quantitative and shows a VAF rounded to the nearest whole percent. The pyrograms here are as follows: **a** wild type control DNA (O% C > T); **b** DNA extracted from a EDTA-blood sample of patient 13,935 (0% C > T); **c** DNA extracted from a tumor sample of patient 13,935 (13% C > T). The pyrogram in figure C shows a G211S variation of 13% VAF. The pyrogram from the blood derived DNA from the same patient is wild type at EP300 codon 211, and the resulting pyrogram is identical to that of the wild type control DNA pyrogram, thus supporting assertions that the G211S variations discussed herein are somatic, and not gremline



The EP300-G211S variant has been described in dbSNP and ClinVar databases as benign (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=rs142030651 and https://www.ncbi.nlm.nih.gov/clinvar/variation/134043/). Furthermore, this variant has been reported in Exac database as a germline variant with 0.5% allele frequency in general and 0.8% in European (non-Finnish) population (http://exac.broadinstitute.org/variant/22-41513727-G-A) [34]. However, our finding showed a much higher frequency (37.2% among TNBC patients). The

Variant Allele Frequencies (VAF) for the G211S variation obtained via both next-generation- and pyro-sequencing were in accordance with VAF values typical for somatic mutations (8.8–13.4%, Table 3) and not VAF approaching those of those expected of a germline mutation (50% or 100% VAFs). To confirm that the EP300-G211S variation was a tumor-specific mutation a random selection of 11 of the G211S-positive patients' genomic DNA was extracted from patients' blood samples and analyzed by pyrosequencing. The sequencing conditions were the same as those of the tumor samples. All of the 11 samples, analyzed in duplicates, showed to contain Glycine in the 211 position (EP300-G211 variant) thus confirming patients' tumor samples positive for G211S were not simply the result of a germline variation.

We found a striking lack of pathogenic mutations typically associated with breast cancer in EP300-G211S positive TNBC cases (Fig. 3a). As mentioned previously, EP300-G211S mutations were only detected in TNBC patients in our cohort. In fact, only two out of 29 TNBC patients with an EP300-G211S mutation had pathogenic somatic mutation in typical breast cancer genes (one single TP53 mutation and one singlee GATA3-mutation). According to a Poisson regression analysis the presence of an EP300-G211S mutation in TNBC patients reduced the presence of any other somatic pathological mutations by 94.9% (regression coefficient: -2.98; $P = 0.005$). When repeating the analysis for TP53 mutations in TNBC patients bearing the EP300G211S-mutation, the regression coefficient was -4.08 (Odds ratio 0.017; $P = 0.0001$).

Furthermore, a survival analysis comparing the classical four main breast cancer subtypes confirmed that this subgroup had a significantly impaired overall survival compared to the other subtypes in our cohort ($P = 0.047$). In addition, we evaluated the association of EP300-G211S mutations with breast cancer specific survival. Following 6.3 years of median follow-up, only 3 of 29 TNBC patients with EP300-G211S mutations present experienced distant relapse, metastasis and death due to breast cancer progression. Conversely, about 20% of the TNBC patients without an EP300-G211S mutation relapsed and had died of breast cancer (Fig. 3a). However, due to the small number of patients, the difference in breast cancer specific survival was not statistically significant ($P = 0.29$).

We found five TNBC patients without any mutations in the investigated genes including EP300. However, two of these patients experienced a relapse including distant metastasis leading to their death (TNBC cases 36 and 41,

Fig. 3a). Other EP300 point mutations, like the V435I substitution, were observed in primary breast cancer tumors belonging to all four major BC subtypes and did not exclude other somatic mutations in typical BC genes as observed for the EP-300-G211S-mutation.

In conclusion, our findings strongly suggest that the presence of an EP300-G211S mutation, observed solely in TNBC patients, correlates with absence of pathogenic somatic mutations in the typical breast cancer associated genes such as TP53, PIK3CA, PTEN, EGFR, and RB1.

Discussion

Our aim in this study was to examine the mutational patterns of breast cancer related genes in primary tumor samples with focus on the triple-negative breast cancer. The study cohort included 86 patients with TNBC along with patients with Luminal A, Luminal B and HER2+ subtype ($n = 149$). We examined the mutational patterns of 44 breast cancer genes using a novel NGS-gene panel. The genetic variations found in the tumor samples were further selected as pathogenic or likely pathogenic according to standards implied by American College of Medical Genetics and Genomics (ACMG) standards. The significance of the mutations was primarily examined using Ingenuity bioinformatics tools [27] and subsequently controlled using COSMIC, SFT, SnpEff, and PolyPhen-2 databases [28, 29, 30, 35].

While typical somatic mutations could be observed in a variety of common breast cancer genes, it became obvious that a subgroup of TNBC patients was genetically distinct, characterized by a nearly complete lack of somatic mutations in typical breast cancer genes. In fact, 38 of the 78 TNBC patients were negative for pathogenic somatic mutations. A subset ($n = 29$) of these patients was, however, positive for a specific mutation in the EP300 gene (Glycine211Serine substitution). Two patients of this subset tested positive for only one single pathogenic mutation each.

The EP300-G211S mutation has not been described in the literature previously to our best knowledge, and its biological and clinical significance remains currently unknown. Interestingly, the EP300-G211S mutation was exclusively found in TNBC tumors. However, other EP300 mutations (such as V435I) could be observed in all BC subtypes and were associated with additional somatic mutations in TP53, PIK3CA, BRCA-1 and other breast cancer related genes (Fig. 3a, b). All EP300-G211S mutations were confirmed by pyrosequencing of

tumor samples. Furthermore, additional pyrosequencing analyses of genomic DNA extracted from patients blood samples indicated that the EP300-G211S substitution was not a germline genetic variation (Fig. 4).

We also investigated a potential association between the G211S-mutation and clinical outcome. Patients who had EP300-G211S mutations in their tumor cells were characterized by a statistically non-significant trend ($P = 0.29$) towards improved overall survival and a reduced risk to develop distant metastasis. Our data indicate that the EP300-G211S mutation may have a role in protecting the TNBC tumors from somatic mutations in cancer-related genes like TP53 and PIK3CA.

EP300, as well as its homologeous protein partner, Creb-Binding Protein (CBP), are histone acetyltranferases which have a significant function in regulation of transcription and modulation of chromatin structure. The EP300/CBP complex facilitates transcription of active genes by acetylating specific lysine residues located at the histone tails resulting in a more open and accessible chromatin structure in the promoter region of target genes. In addition, the EP300/CBP complex also functions as a bridge linking the basal transcription components to the sequence-specific transcription factors [36]. The involvement of EP300 and CBP in critical signaling pathways mediated by TP53 [37], BRCA1 [38], and nuclear receptors [39] explains how their inactivation may contribute to the initiation of cancer.

Pathogenic mutations in EP300, as well as CBP, are considered to be rather rare [24, 40] and, thus, have not received much attention in the literature for this reason. Pathogenic mutations are mostly found on the HAT domain of the two proteins and the mutation hotspots are limited to a few amino acid residues surrounding the acetyl-CoA binding site [40]. Our search in the COSMIC (Catalogue of Somatic Mutations in Cancer) database revealed that pathogenic mutations in the extreme N-terminal of the EP300 gene are much less frequent than of other regions (less than 2 counts per mutation).

Postulating an underlying mechanism for the phenotypical differences between G211S positive and negative TNBC patients has proven challenging. However, EP300 nonsense mutations resulting in truncated proteins have been identified in some epithelial primary cancers suggesting that EP300 may function as a classical tumor suppressor gene [41]. In this study we found only one TNBC tumor sample bearing EP300 truncating mutations that at the same time

contained pathogenic mutations in several other relevant genes. In addition to the mentioned G211S substitution, two other mutations in EP300 were found to be relatively frequent, V435I and Q2223P. The latter mutation was specific for TNBC in our material and only found in combination with G211S mutations. The double mutants exhibited the same genotype as G211S single mutants, i.e., they were exclusively free of pathogenic mutations in other breast cancer genes (Fig. 3a), indicating that Q2223P mutations probably have no profound effect on the outcomes of the G211S mutation. This observation reinforces to some extent our hypothesis that the EP300-G211S mutation is a specific molecular signature on its own. Q2223P single nucleotide polymorphisms (SNP) have been described in familial breast cancer without any significant effect on the disease [42].

The amino acid residue Glycine-211 is located in the N-terminal of EP300 in a structurally undefined region of the protein. Glycine-211 and its surrounding amino acid residues may be part of an intrinsically disordered region of EP300 providing flexibility for interaction with transcription factors. For example, EP300 binds the intrinsically disordered N-terminal transcriptional activation domain TP53 with varying affinities at least four domains of EP300 (TAZ1, TAZ2, KIX, and NCBD; see also Fig. 1 for details) suggesting multivalent binding where each individual activation domain of TP53 may interact with different domains of EP300 [43, 44, 45].

EP300 is a relatively ancient protein that diverged from its paralog, CBP, via gene duplication prior to vertebrate radiation approximately 450 million years ago [46]. While the functional domains of vertebrate of EP300, such as TAZ, KIX, Bromo, PHD, and HAT, show high percentage of identity of the amino acid level, the N-terminal part shows much lower homology. This observation is in favor of the N-terminal of EP300 possessing an intrinsically disordered region. Therefore, it can be argued that a simple substitution mutation in this region such as G211S may not have a profound effect on the structure and function of the EP300 protein. However, it should be noted that the G211S mutation may cause enhanced phosphorylation at the position of the newly acquired serine residue. For example, others have shown that phosphorylation of EP300 at a single site (serine 89) had an inhibitory effect on the transcriptional activity of the protein [47]. On the contrary, phosphorylation of EP300 at Serine 1834 was essential for HAT activity [48]. Alternatively, as EP300 is known to be an important nuclear transcriptional co-factor of estrogen receptor alpha (ERα) [49], one might speculate that the G211S mutation may be

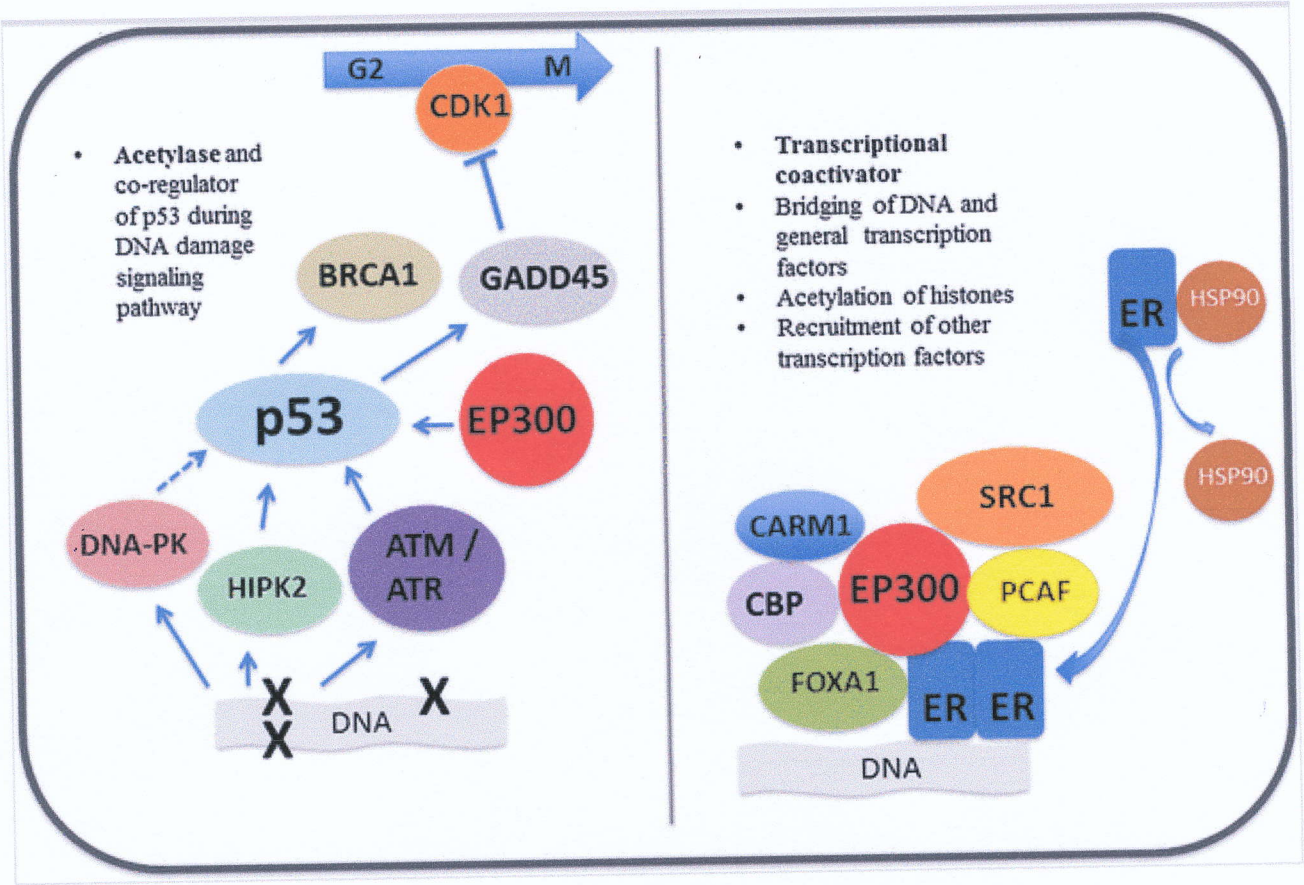
involved in down-regulation of the ERα by so far unknown mechanisms. This hypothesis will be further investigated in currently ongoing preclinical studies.

Finally, it has been postulated that EP300 is targeted by numerous viral proteins [25]. It is well-established that virus infections may be a precursor of malignant diseases. Thus, it has been shown that adenovirus E1A is able to recruit EP300 and the retinoblastoma protein pRb into a ternary complex promoting acetylation and degradation of pRb and loss of cell cycle control [50, 51]. The critical role of CBP and EP300 during the regulation of cell signaling and transcription makes them potent targets for viral proteins. Interestingly, concerning triple-negative breast cancer, detection of high-grade human cytomegalovirus (HCMV) protein expression has recently been associated with a down-regulation of both ER-α and progesterone receptor levels in human breast cancer samples [52]. The expression of HER-2 was also reduced in HCMV-positive samples, however, without reaching the level of statistical significance. It is hence possible that viruses through interaction with the mutated form of EP300 are debilitated to recruit and degrade Rb, which may reduce negative effects on cell cycle control, thereby providing a better prognosis for these patients.

In summary, the precise mechanisms behind EP300's contribution to the observed genotype in TNBC patients are currently unknown. However, due to the central role of EP300 in regulation and integration of transcriptional and signaling pathways, including steroid receptor signaling and regulation of P53 activity (Fig. 5), it is a promising target for ongoing research aiming at resolving the biology of triple-negative breast cancer.

Fig. 5

Selected pivotal roles of p300/EP300 in human breast cancer cells: The two major roles of EP300 (acetyltransferase and transcriptional co-activator) are shown including the key genes involved. ATM, ataxia-telangiectasia mutated gene; CARM1, co-activator-associated arginine methyltransferase 1; CBP, CREB-binding protein; CDK1, cyclin-dependent kinase 1; ER, estrogen receptor; FOXA1, forkhead box protein A1; GADD45, growth arrest and DNA damage 45 gene(s); HIPK2, homeodomain-interacting protein kinase 2; HSP90, heat shock protein 90; PCAF, p300/CBP-associated factor; SRC1, steroid receptor co-activator 1



In conclusion, the mutational status of EP300 may potentially be used as a surrogate marker for the overall mutational status in TNBC patients and serve as a potential prognostic marker in this subgroup of patients.

Acknowledgements

The authors thanksfully acknowledge Ms. Gro Gundersen, Ms. Eva Smedsrud and M.Sc. Torben Lüders for the preparation of genomic DNA from FFPE samples (G.G. & E.S.) and EDTA-samples prior to pyrosequencing (T.L.).

Funding

This study was funded by Bodil and Magne´s Cancer Research Fund, Oslo, Norway.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Ethical approval All procedures performed in this study were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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