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Full sequencing of *TP53* identifies identical mutations within *in situ* and invasive components in breast cancer suggesting clonal evolution

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ABSTRACT

In breast cancer, previous studies have suggested that somatic TP53 mutations are likely to be an early event. However, there are controversies regarding the cellular origin and linear course of breast cancer. The purpose of this study was to investigate tumor evolution in breast cancer by analyzing TP53 mutation status in tumors from various stages of the disease. The entire coding sequence of TP53 was sequenced in a cohort of pure ductal carcinoma *in situ* (DCIS), pure invasive cancer (≤ 15 mm) and mixed lesions (i.e. invasive cancer with an *in situ* component). Of 118 tumor samples, 19 were found to harbor a TP53 mutation; 5 (15.6%) of the pure DCIS, 4 (10.5%) of the pure invasive cancers and 10 (20.8%) of the mixed lesions. In the mixed lesions, both the invasive and the DCIS components showed the same mutation in all 5 cases where the two components were successfully microdissected. Presence of the same mutation in both DCIS and invasive components from the same tumor indicates same cellular origin. The role of mutant TP53 in the progression of breast cancer is less clear and may vary between subtypes.

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1. Introduction

TP53 is a key tumor suppressor gene with a potentially large clinical impact (Vousden and Lane, 2007). It encodes the tumor suppressor protein p53, which prevents tumorigenesis by controlling cell cycle regulation, apoptosis, DNA repair and the surveillance of genomic integrity (Ko and Prives, 1996; Levine et al., 1991; Oren, 1999; Vousden, 2000). TP53 is the most commonly mutated gene in human cancers (Hainaut and Hollstein, 2000). The majority of mutations occurs in the core domain, the most conserved area of the

gene, which may interfere with the protein–DNA interaction or affect the stability of the 3D structure, and lead to the synthesis of an abnormal version of the p53 protein. In breast cancer the frequency of TP53 mutations ranges from 20 to 50% (Powell et al., 2000). Previous studies suggested that abrogation of TP53 function might accelerate the development of a genetic instability phenotype leading to a heterogeneous breast cancer (D'Assoro et al., 2008; Medina et al., 2003).

Breast cancer has long been recognized as a heterogeneous disease with varying clinical outcome. Breast cancer

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development and progression is a multistep process that manifests through a series of pathological stages, namely atypical ductal hyperplasia, ductal carcinoma *in situ* (DCIS), invasive carcinoma and eventually metastatic carcinomas (Allred et al., 2001; Polyak, 2008). Models of breast tumor progression, however, have been discussed for many years, and it is not clear which cell population could progress to invasive disease. Specific molecular markers may help us to understand more about the clonality of the tumor.

TP53 mutation might be an early event occurring in DCIS or even earlier than DCIS stage (Done et al., 2001a; Ho et al., 2000; Kang et al., 2001). Moreover, molecular and pathological evidence suggests that DCIS can be a precursor to invasive disease (Claus et al., 2001; Hwang et al., 2004; Ma et al., 2003). Therefore, investigating the mutation status of TP53 in both the DCIS and the invasive components in the same lesion could be a way to study the progression of breast cancer.

In the current study, we investigated the status and timing of TP53 mutations in the progression from DCIS to invasive breast cancer. Cases of pure DCIS, pure invasive cancer and mixed lesions were explored as well as both DCIS and invasive components of the mutated mixed cases.

2. Material and methods

2.1. Study cohort

The study cohort is a population-based cohort, including all 854 women who were diagnosed with either a pure DCIS, a pure invasive breast cancer (≤ 15 mm) or a mixed lesion (i.e., invasive carcinoma with *in situ* component) between 1986 and 2004 in Uppland, Sweden. Of the 854 women, 258 had frozen tumor material prospectively preserved in the biobank at Uppsala University Hospital. In this study, we included 118 of the 258 women with sufficient tumor material, which comprised all 32 with pure DCIS (27.1%), all 38 (32.2%) with pure invasive breast cancer and a random sample of 48 with mixed lesions (40.7%). This study has been approved by the Ethics Committee at Uppsala University Hospital (Dnr 2005:118).

All DCIS cases were carefully looked at by a pathologist, and no invasive component was found in the specimen. All histopathological specimens, both paraffin embedded and frozen, were evaluated twice by two different pathologists. Invasive breast cancer was classified based on the Elston–Ellis classification system, grade I–III (Elston and Ellis, 1993). DCIS lesions were classified according to the European Organization for Research and Treatment of Cancer (EORTC) system (Holland et al., 1994). In this study we denoted the DCIS grades A–C (corresponding to grade I–III) to emphasize that DCIS and invasive lesions were classified based on different systems. In lesions with both an invasive and an *in situ* element, the classification was done in both elements separately.

2.2. Cryosectioning and laser capture microdissection

Ten sections from each frozen sample at 20 μ m thickness were prepared with a cryotome; the sections were placed in

Eppendorf tubes and immediately stored at -80°C for DNA extraction.

In the mixed lesions with TP53 mutations, the DCIS and invasive components were separated by microdissection. The samples were cryosectioned at both 4 μ m and 14 μ m thicknesses. The 4 μ m-thick section was stained by routine H&E staining to locate the corresponding areas to be microdissected in the consecutive 14 μ m-thick section. The 14 μ m-thick sections were mounted on a slide pre-covered with a thin polyethylene membrane (PALM slide) and immediately stored at -80°C until microdissection.

Laser capture microdissection (LCM) was performed on frozen sections using a Zeiss inverted microscope PALM Laser Micro-Beam System (Carl Zeiss, German). Cryosected sections were thawed for 30 s and immediately stained using 60 μ L hematoxylin (mixed with RNasin) for 1 min, incubated in 60 μ L Zincofix for 30 s and followed by 30-s incubation steps in 75%, 95% and 100% ethanol, respectively. Slides were air-dried and kept desiccated to be dissected. Under light microscopic examination we microdissected as close to 3000 cells as possible from different parts of the same component. The *in situ* carcinoma cells and/or invasive carcinoma cells were captured into the collecting caps, preserved in 50 μ L Trizol and immediately stored at -80°C for DNA extraction.

2.3. DNA extraction and TP53 mutation analysis

DNA was isolated from whole sections using chloroform/phenol extraction followed by ethanol precipitation (Nuclear Acid Extractor 340A; Applied Biosystems) according to standard procedures. The microdissected cells were manually purified for Trizol using chloroform/phenol extraction. The DNA containing fraction was ethanol precipitated and dissolved in 100 μ L ddH₂O, incubated at 37°C overnight in 100 μ L buffer (10 mM Tris (pH 8.0), 1 mM EDTA (pH 8.0), 1% Tween-20, and 1 mg/mL proteinase K) and heat-inactivated at 95°C for 10 min to deactivate the proteinase K. A volume of 5 μ L of cell lysates was used for polymerase chain reactions (PCRs).

The entire coding sequence of the TP53 gene (exons 2–11) was analyzed for mutations by sequencing using the 3730 DNA Analyzer (Applied Biosystems, USA). All fragments were sequenced in both directions and the PCR primers used were linked to universal M13 sequences (Supplementary data; Table 3). DNA (25 ng) was amplified in a 10 μ L reaction using HotStarTaq DNA Polymerase (Qiagen) and touchdown PCR with annealing temperatures from 68°C to 56°C (MJ Tetrade, BioRad). The PCR products were purified with MultiScreen[®] PCR μ 96 Plate (Millipore) on epMotion[™]5075 (Eppendorf) and the quality checked on a 1.5% agarose gel. BigDye Terminator reaction mix v1.1 (Applied Biosystems) was used in the sequencing reaction, and the products were purified with Sephadex[®] G-50 Superfine (Qiagen).

SeqScape[®] Software v2.5 (Applied Biosystems) was used for alignment to reference sequence and the scoring of mutations were carried out by two operators independently. GeneBank accession number NM_000546 was used as reference sequence.

2.4. Statistical analysis

Pearson Chi-square (χ^2) test and Fisher's exact test were performed to study the frequency distribution of selected variables. Statistical significance threshold was set to $P \leq 0.05$. All calculations were performed using the R (www.r-project.org).

3. Results

3.1. TP53 mutations

The results of the mutation analysis of exons 2–11 of TP53 in the 118 breast tumors were stratified into three distinct diagnosis groups (pure DCIS, pure invasive cancers, or mixed diagnosis), and altogether 19 TP53 mutations (16.1%) (Table 1) were detected. The majority of mutations (14/19, 73.7%) were distributed in exons 5–8, corresponding to the core DNA binding domain of the p53 protein. A large fraction of the mutations were missense (10/19, 52.6%), whereas 4 (21.1%) were non-sense, 4 (21.1%) were frameshift, and one mutation was at an intron–exon boundary (Table 1).

3.2. Comparison of the TP53 mutations between pure DCIS, IDC and mixed lesions

The proportion of mutated TP53 in the mixed-lesion group was slightly higher (10/48, 20.8%) than in the DCIS group (5/32, 15.6%) and the pure invasive group (4/38, 10.5%),

although not statistical significant ($P = 0.46$). The proportion of TP53 mutation in the DCIS group (5/32, 15.6%) was almost equal to that in the combined invasive group (the pure invasive group plus mixed-lesion group, 14/86, 16.3%).

Moreover, no significant difference was found between the three groups in terms of the position (codon, exon) of the TP53 mutations or the location of missense mutations within or outside the DNA binding motif (DBM). With regard to the nature of the mutation (base change, deletion, insertion) the DCIS group seems to only have single base alterations. Concerning the predicted effect of the mutation (missense, non-sense, frameshift, in-frame, splice) the pure DCISs harbored missense mutation (4/5, 80%) more frequently than pure invasive cancers and mixed lesion combined (6/14, 43%), although not statistically significant ($P = 0.30$). All mutations have been previously reported and the fraction of hotspot versus rare mutations did not vary between the three groups (<http://www-p53.iarc.fr/>; Petitjean et al., 2007).

3.3. TP53 mutation status explored in mixed-lesion samples

To explore the timing of the development of a TP53 mutation during breast cancer progression, the ten mixed cases found to harbor mutated TP53 from the first round of sequencing were microdissected. From five of the ten TP53 mutated mixed cases, we successfully microdissected cells from both cell compartments (invasive and DCIS), which revealed high DNA quality. The same TP53 mutation detected in the bulk

Table 1 – TP53 mutations in 19 samples detected by DNA sequencing.

Case no.	Exon	Codon	Nucleotide	Effect	Location of missense mutation
<i>Pure DCIS</i>					
FW06-05	7	245	GGC → AGC	Missense	DBM ^c
FW06-67	6	220	TAT → TGT	Missense	Non-DBM
FW06-100	10	360	GGG → GCG	Missense	Non-DBM
FW06-142	6	213	CGA → TGA	Nonsense	
FW06-183	7	235	AAC → AGC	Missense	Non-DBM
<i>Pure invasive</i>					
FW06-68	5	133	ATG → AAG	Missense	DBM
FW06-141	6	216	GTG → ATG	Missense	Non-DBM
FW06-145	8	301	Del C	Frameshift	
FW06-174	6	213	CGA → TGA	Nonsense	
<i>Mixed lesions</i>					
FW06-30 ^a	10		IVS10-1 G → A	Splice	
FW06-38 ^a	6	220	TAT → AAT	Missense	Non-DBM
FW06-65 ^a	4	106–109	10 bp del	Frameshift	
FW06-69 ^a	6	192	CAG → TAG	Nonsense	
FW06-88 ^b	7	235	AAC → AGC	Missense	Non-DBM
FW06-112 ^b	8	306	CGA → TGA	Nonsense	
FW06-164 ^b	4	47	Del C	Frameshift	
FW06-208 ^b	4	83	Del G	Frameshift	
FW06-219 ^a	7	248	CGG → TGG	Missense	DBM
FW06-236 ^b	7	257	CTG → CAG	Missense	Non-DBM

a The TP53 mutation was detected in the invasive component; missing data from the DCIS component.

b The same TP53 mutation was found in both DCIS and invasive components.

c DBM: DNA-binding motifs (Olivier et al., 2006).

tumor, was seen both in the DCIS and the invasive components of the same tumor (Table 1).

3.4. Associations between TP53 mutations and clinicopathological characteristics

The relationship between TP53 gene mutations and the clinicopathological characteristics is shown in Table 2. In these analyses, the pure invasive and the mixed cases were combined in one group. TP53 mutation status was statistically significantly associated with age at diagnosis ($P = 0.05$), ER status ($P = 0.0008$), PR status ($P = 0.03$) and proliferation (by Ki67; $P = 0.02$). TP53 mutation status was not correlated with histopathological grade of the DCIS tumors. However, the invasive tumors displayed strong association between TP53 mutations and histopathological grade ($P = 0.007$). Furthermore, no statistical significance could be attributed to lymph node status or HER2 status (Table 2).

4. Discussion

In the current study, 19 mutations were detected in the DCIS, pure invasive and mixed-lesion groups. The sequencing analysis detected 26% of the TP53 mutations outside exons 5–8, pointing to the importance of analyzing the whole gene and not merely exons 5–8 as performed in most previous studies. DNA sequencing in both directions minimized scoring of false positive and false negative mutations. The overall fraction of samples with mutated TP53 (16.1%) is lower than that in the average breast cancer series (25.0%) (<http://www-p53.iarc.fr/>), and may be due to the small sized lesions in our study.

From the population-based cohort, only a subset of cases with sufficient frozen tissue was available. The cases not included were most likely impalpable or very small tumors of which the biobank did not include enough frozen material. Given that tumor size is highly related with tumor grade, the cases not included in this study should be comprised of an overrepresentation of low-grade tumors. Moreover, TP53 mutation is more likely to occur in higher grade tumor (Done et al., 2001b; Simpson et al., 2001). Thus, we might have overestimated the true proportion of TP53 mutations. This overestimation was unlikely to be larger in DCIS than in the invasive cancer.

Immunohistochemistry (IHC) has been used in a number of studies to detect mutated p53 protein. This is partly based on the accumulation of mutant protein in the cell due to lack of MDM2 binding that is needed for the degradation of the protein. However, about 30% of mutations detected by sequencing are missed using IHC (Sjogren et al., 1996). The sensitivity of mutation detection using DNA sequencing is higher today than the sensitivity in earlier papers reporting on TP53 mutation status. We have previously investigated the sensitivity for DNA sequencing to be 10–15% allele sensitive (data not shown), which means that about 10–15% mutated tumor cells are sufficient to detect mutated TP53. By only sequencing exons 5–8, 5–20% of the mutations would not be detected. Some studies (Done et al., 2001a; Kang et al., 2001) have reported on the timing of TP53 mutational events

Table 2 – Associations between TP53 mutations and clinicopathological characteristics.

Characteristics	Total number	TP53 mutation no. (%)	P
Number of cases	118	19 (16.1)	–
Age (year)			0.05
≤55	48	12 (25.0)	
>55	70	7 (10.0)	
Tumor grade			0.19
DCIS	32	5 (15.6)	
A	1	1 (100.0)	
B	15	1 (6.67)	
C	16	3 (18.8)	
Invasive cancer	86	14 (16.3)	0.007
I	32	3 (9.38)	
II	42	5 (11.9)	
III	12	6 (50.0)	
Lymph node status ^a			0.93
Negative	18	4 (22.2)	
Positive	65	14 (21.5)	
Unknown	3	1 (33.3)	
ER status ^b			0.0008
Negative	25	10 (40.0)	
Positive	93	9 (9.7)	
PR status ^b			0.03
Negative	34	10 (2.9)	
Positive	84	9 (10.7)	
HercepTest ^{®c}			0.56
Negative	39	8 (42.1)	
Positive	79	11 (13.9)	
Ki 67			0.02
Positive	82	10 (12.2)	
Negative	26	9 (34.6)	

a DCIS cases were not included in the group.

b ER, PR and Ki67 scoring ≤10% is regarded as negative; scoring >10% is regarded as positive.

c HercepTest scoring 0 + I is regarded as negative; scoring II + III is regarded as positive.

based on sequencing exons 5–8, so it is necessary to sequence the whole gene of TP53 to evaluate the progression of breast cancers.

The proportion of samples with TP53 mutation was slightly higher in the mixed-lesion group than in the DCIS and the pure invasive groups. However, larger and more advanced cases were included in this group. Furthermore, the proportions of TP53 mutations in DCIS and the invasive group (pure invasive plus mixed lesion) were almost equal, suggesting that TP53 mutation might be an early event occurring at or prior to the DCIS stage. Still, the frequency of mutation was not found to statistically significantly vary between the DCIS, the pure invasive cancer and mixed-lesion groups.

The objective of this study was to characterize the timing of TP53 mutation in the progression of breast cancer. For this purpose, we investigated the DCIS and the invasive components

synchronously. In 5 out of 10 mixed lesions with TP53 mutation, both DCIS and invasive components were successfully microdissected. Interestingly, in those mixed-lesion samples, the same mutation found in the DCIS component was also observed in the adjacent invasive component. This finding suggested that TP53 mutation would occur before invasion, at the DCIS or prior to the DCIS stage during the progression of breast cancer, which agrees with previous reports on TP53 mutation (Done et al., 2001a; Ho et al., 2000; Kang et al., 2001). The same mutation detected in the two different components of the same tumor also indicated that the DCIS and invasive cells arose from the same tumor cell clone.

Subclassification based on gene expression profiles (Muggerud et al., unpublished data) showed that these mixed tumors with TP53 mutation in both components are of various molecular subtypes (normal-like, basal-like, luminal B and ERBB2⁺), suggesting that clonal evolution is not exclusive for one subtype. As expected, a high fraction of basal-like tumors were observed among mutant samples (7/18), but the different subtypes did not show significantly skewed distribution between the DCIS, pure invasive and mixed-lesion groups with TP53 mutation.

In breast cancer, different types of TP53 mutations have been reported with different impact on survival and chemoresistance (Aas et al., 1996; Borresen-Dale, 2003; Borresen et al., 1995; Olivier et al., 2006). We observed a higher frequency of missense mutations in the DCIS compared to the invasive and mixed groups, which showed a higher frequency of non-missense mutations (mostly frameshift as well as non-sense and splice mutations). One could hypothesize that the mutations in DCIS would be of a milder and less deleterious type that still had not caused invasion. Two mutations in the DCIS (G245S and Y220C) have previously been associated with better survival compared with any other missense mutation (Olivier et al., 2006), supporting the theory. The numbers are, however, too low to allow any conclusion.

In invasive cancer, TP53 mutation frequency correlated with histopathological grade. Similar to earlier studies (Done et al., 2001b; Simpson et al., 2001), we found more mutations in high-grade DCIS lesions than in low- or intermediate-grade DCIS lesions but this was not statistically significantly different in this small cohort. According to the linear model of breast cancer progression, low-grade DCIS more likely progress to low-grade invasive cancer and high-grade DCIS progress to high-grade invasive cancer.

In conclusion, TP53 mutation is likely an early event in breast cancer, occurring prior to or at the *in situ* stage. Presence of the same mutation in both DCIS and invasive components from the same tumor indicates the same cellular origin. The role of mutant TP53 in the progression into invasive cancer is still unclear and may vary between subtypes of breast cancer.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at [doi:10.1016/j.molonc.2009.03.001](https://doi.org/10.1016/j.molonc.2009.03.001).

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