Differential Immunohistochemical and Biological Profile of Squamous Cell Carcinoma of the Breast

JULIEN GRENIER[1,2], JEAN-CHARLES SORIA[1], MARIE-CHRISTINE MATHIEU[3], FABRICE ANDRE[1], SELMA ABDELMOULA[3], VALERIE VELASCO[3], LUC MORAT[4], BENJAMIN BESSE[1], ARIANE DUNANT[5], MARC SPIELMANN[1] and SUZETTE DELALOGE[1]

[1]Department of Medical Oncology, [3]Department of Pathology and [5]Department of Biostatistics, Institut Gustave Roussy, 39 rue Camille Desmoulins, 94805 Villejuif; [2]Department of Medical Oncology, Hôpital Henri Mondor, 51 avenue du Maréchal de Lattre de Tassigny, 94000 Créteil; [4]Commissariat à l'Énergie Atomique (CEA), DSV/DDR/LRO, 18 route du Panorama, 92265 Fontenay aux Roses, Cedex, France

Abstract. *Background: Pure or metaplastic squamous cell carcinoma (SCC) of the breast is a rare entity with an unclear pathogeny and aggressive clinical behaviour. An attempt was made to characterize its differential immunohistochemical and biological profile. Patients and Methods: Twenty-seven cases of SCC (pure or not) of the breast were matched with 27 ductal invasive carcinomas (IDC) for age, tumour size, nodal involvement and year of diagnosis. The expression levels of oestrogen receptor (ER), progesterone receptor (PR), Ki-67, epidermal growth factor receptor (EGFR), HER2, Cyclin B1, hTERT, cytokeratins (CK) 5/6 and p63 were determined immunohistochemically in both cohorts. The presence of the human papilloma virus (HPV) genome was investigated by polymerase chain reaction (PCR). Results: Pure and metaplastic SCC displayed common profiles typifying a basal origin: they never expressed ER or PR, were HER2-negative in 93% of cases, exhibited positivity for CK5/6 or EGF-R in 75% and 85%, and for p63 in 70% of cases and were highly proliferative. These profiles were markedly different from those of matched controls ($p<0.001$ for five markers) except for HER2 and hTERT. The HPV genome was detected in 2 out of 14 cases (14%) of SCC. Conclusion: The expression profile of SCC of the breast was markedly different from that of IDC. A typical "basal-like" phenotype was displayed that may explain part of their behaviour and justify specific therapeutic approaches. HPV infection was not a leading oncogenic event in SCC of the breast.*

Correspondence to: Dr. Julien Grenier, Department of Medical Oncology, Hôpital Henri Mondor, 51 avenue du Maréchal de Lattre de Tassigny, 94000 Créteil, France. Tel: +33 1 49 81 25 78, Fax: +33 1 49 81 25 69, e-mail: julien.grenier@hmn.aphp.fr

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Recent data derived from micro-array analyses and extended to immunohistochemical classifications have tended to subclassify breast carcinomas, according to their expression of a subset of markers, into at least six categories: Her-2 overexpressing, basal-like, luminal type A, B and C, and normal-like breast cancers (1-3). Further data have shown that these newly described groups may display very different clinical behaviours and sensitivity to drugs (4, 5). Such classification therefore appears to be a potentially promising tool for therapeutic targeting of localized or advanced breast cancer.

Pure squamous cell carcinoma (SCC) of the breast is a very rare malignancy. It accounts for less than 0.1% of all breast carcinomas, while carcinoma with squamous metaplasia accounts for 3.7% (6-11). The pathology of SCC of the breast remains unclear although it has been suggested that it may originate from squamous metaplasia of the ductal epithelium. However, squamous metaplasia may arise from myoepithelial cells (12).

These tumours are rather aggressive (10). Most of them are negative for estrogen (ER) and progesterone (PR) receptors, but a detailed immunohistochemical profile is unknown. In an attempt to better characterise and understand this particular entity and to determine whether or not it could be classified within one of the particular breast cancer subgroups recently described, the pathological and biological features of pure or largely metaplastic squamous cell carcinoma of the breast were analysed. These tumours were compared to 27 matched cases of invasive ductal carcinoma (IDC) by immunohistochemistry, for the expression of ER and PR, epidermal growth factor receptor (EGFR),

Table I. *Antibodies and methods used for immunohistochemistry.*

Antigen	Antibody clone	Dilution	Incubation	Antigen retrieval
ER	6F11 (Novocastra)	1/30	Horse serum 5% 60 min	Micro-wave 30 min
PR	PgR 636 (Novocastra)	1/120	Horse serum 5% 60 min	Micro-wave 30 min
Ki-67	MIB-1 (Dako)	1/50	Horse serum 5% 20 min	Micro-wave 30 min
EGFR	3197 (Zymed)	1/100	Primary antibody 32 min	Protease
HER2	A0485 (Dako)	1/1000	Primary antibody 45 min	Micro-wave 30 min
Cyclin B1	NCLB1-7A9 (Novocastra)	1/60	Primary antibody 60 min	Micro-wave 3 min
hTERT	NCL44-F12 (Novocastra)	1/50	Horse serum 2.5% 12 H	Boiling 30 min
CK 5/6	D5/16B4 (Chemicon)	1/1600	Horse serum 2.5% 20 min	Micro-wave 18 min
p63	4A4 (Neomarker/Microm)	1/50	Primary antibody 30 min	Micro-wave 30 min

HER2/neu, Cyclin B1, Cytokeratins (CK) 5 and 6, Ki-67, hTERT and p63. These markers, which also represent key oncogenic pathways, enable the subclassification of breast cancers into the categories described above (1, 13). In addition, in order to explore the possibility that the human papillomavirus (HPV) might be involved in the pathogenesis of squamous cell carcinoma of the breast, the presence of HPV DNA in these tumours was investigated by polymerase chain reaction (PCR).

Patients and methods

Patients. The charts of all consecutive patients diagnosed with pure SCC or carcinoma with squamous metaplasia at the Institut Gustave Roussy, between 1981 and 2004, were reviewed. Thirty-seven patients were identified in our database. Ten patients were excluded from the analysis for the following reasons: misclassified infiltrative apocrine carcinomas (n=2), squamous cell carcinoma of the skin (n=1) and insufficient tissue for the immuno-histochemical assay (n=7). Twenty-seven cases were therefore available for a complete analysis. Patient data including age, clinical and pathological features, stage, treatment and outcome were collected retrospectively. For each case, one control with a diagnosis of ductal invasive carcinoma was selected and matched for age at diagnosis, tumour size, axillary lymph node status, and year of diagnosis.

Pathology and immunohistochemistry. All the slides of SCC were independently analyzed by two pathologists specializing in breast cancer. The carcinomas were considered pure SCC if the tumours exhibited predominantly squamous differentiation, no glandular differentiation, keratinizing cells and large cells. If the tumours harboured areas of squamous cells with areas of undifferentiated carcinoma or, glandular differentiation or ductal carcinoma *in situ*, they were considered as carcinoma with squamous metaplasia.

It was decided that histological grading of the tumour was not relevant in this subtype of breast cancer due to the absence of a glandular component in the great majority of cases.

Formalin-fixed, paraffin-embedded 4 μm-thick tissue sections were immunohistochemically stained for each primary antibody, as specified in Table I. The slides were dewaxed in xylene and hydrated through graded ethanol (100%, 95%, and 70%). To retrieve the antigenicity, the slides were brought to the boil over 20 to 30 min with citrate buffer ph 6.0 to 7.3. Then, endogenous peroxydase activity was blocked by a 3% H_2O_2 solution for 5 to 20 min. The sections were blocked in normal horse serum 2.5 or 5% and then incubated with the primary antibody according to the conditions specified in Table I. The reaction was revealed by the avidin-biotin-complex-peroxydase technique (ABC, Ventana, Tuscon, USA) using diaminobenzidine (DAB, DAKO) as the chromogen, (For EGFR, hTERT and CK 5/6) or with the secondary antibody (ENVISION system, DAKO, Copenhague, Danemark) (for ER, PR, Ki-67, HER2, P63 and Cyclin B1) and revealed using diaminobenzidine (DAB, DAKO, Copenhague, Danemark) as the chromogen. Slides were rapidly counterstained with hematoxylin and evaluated microscopically. The labelling index and cut-off for positivity of each antibody are defined in Table II.

P63 was the latest marker analyzed for refinement of the subclassification. Analyses could only be performed in 17 of the cases of SCC and 18 of the cases of invasive ductal carcinomas because of insufficient residual tissues.

HPV detection by PCR. DNA was extracted from two paraffin-embedded, 4-μm-thick tissue sections. All of the sections were deparaffinized using a series of xylene baths and then rehydrated using a graded alcohol series. Samples were digested with proteinase K and subjected to extraction with phenol followed by extraction with chloroform/isoamyl alcohol. The extracted DNA was precipitated with ethanol and the pellet was resuspended in 30 μL nuclease-free water. HPV positive cervical carcinoma sections were used as positive controls. The PCR was carried out as previously described, with the L1 primer pair MY09/MY11 in a final reaction volume of 100 μL containing 5 μL of genomic DNA (20). The DNA was migrated on an agarose gel, stained with ethidium bromide, and photographed under UV light. The quality of the isolated DNA was tested by PCR amplification of the β-globin gene.

Statistical analyses. The results are expressed as means (± standard deviations) for quantitative data. For qualitative data, percentages were computed for available values. The χ^2 test was used for comparisons between pure and non-pure squamous cell carcinoma. The cases and controls were compared with linear models for quantitative data and McNemar's test for proportions (21). All analyses were performed with SAS® (version 8.2; SAS Software, Cary, NC, USA).

Table II. *Labelling indices and positivity cut-offs of antibodies.*

Antigen	Intensity	Cut-off for positivity
ER/PR	1+: slightly positive 2+: moderately positive 3+: strongly positive	≥10% of cells stained, any intensity (14)
Ki-67		≥20% cells stained (15)
EGFR	1+: slightly positive 2+: moderately positive 3+: strongly positive	≥10% of cells stained, any intensity (16)
HER2	0: lack of stain or <10% of cells stained 1+: slight and incomplete membrane staining or >10% of cells stained 2+: moderate and complete membrane staining of >10% of tumour cells (FISH required) 3+: strong and complete membrane staining of >10% of tumour cells	Score 3+ only (17)
Cyclin B1	Any intensity	≥5% of cells stained (18)
CK 5/6	Any intensity	≥1% of cells stained (16)
hTERT	Grade 1: no staining or cytoplasmic only Grade 2: 1-10% positive nuclei or nucleoli Grade 3: 10-50% positive nuclei or nucleoli Grade 4: >50% positive nuclei or nucleoli	Grade 3 and 4 only (19)
p63	Any intensity	>1% of cells stained

Table III. *SCC and IDC patient characteristics.*

Characteristics	SCC	IDC
N	27	27
Age, mean		
years	55 (31-85)	55 (31-86)
Tumour size, mean		
mm	34 (5-120)	29 (10-60)
T0-T1-T2	22 (81%)	23 (85%)
T3-T4	5 (19%)	4 (15%)
Lymph node status		
Positive	11	11
Negative	12	14
Unknown	4	2

SCC: squamous cell carcinoma; IDC: invasive ductal carcinoma.

Results

Patient characteristics. The characteristics of the SCC patients and controls were well matched. They are summarised in Table III and histological results are shown on Figure 1.

Eleven patients had pure SCC while sixteen patients had carcinomas with squamous metaplasia. Keratinization was found in 23 tumours. In the majority of cases, the stromal reaction consisted of lymphocytes and, in one case, there was spindle cell metaplasia. None of the neoplasms had cartilaginous or osseous metaplasia. Eight tumours were cystic.

Recurrence, follow-up and outcome. In the cohort of patients with SCC, at a median follow-up of 5.5 years (range 3.8-6.6 years) the 5-year disease-free survival (DFS) rate was 52% and the overall survival (OS) rate was 57%. In the matched group, at a median follow-up of 3.8 years (range 3.2-5.4 years), five-year DFS and OS were 76% and 84%, respectively.

Immunohistochemical profile and HPV detection. The results of the immunohistochemical studies are summarized in Table IV and shown in Figure 2. None of the SCC expressed estrogen or progesterone receptors, while 80% of invasive ductal carcinomas were ER positive and 64% were PR positive ($p<0.0001$). The Ki-67 index was more frequently elevated (over 20% in 77% of cases) in SCC than in ductal invasive carcinomas ($p=0.017$). EGFR was overexpressed in 85% of SCC *versus* only 12% of ductal invasive carcinomas ($p=0.0041$). Similarly, Cyclin B1 was overexpressed in 60% of the SCC compared to 16% of the controls ($p=0.0001$). Cytokeratins 5/6 were overexpressed in 75% of SCC *versus* 0% in controls ($p=0.0006$) and P63 was overexpressed in 70% of SCC *versus* 11% in controls ($p=0.11$). HER2 and hTERT expression did not differ between the two populations. The expression profiles were not different between pure and metaplastic carcinomas of the breast (Table V). Overall, the expression profile of pure or metaplastic squamous cell carcinomas was typical of the profiles described as "triple-negative" "basal-like" breast tumours (Table VI).

Fourteen of the SCC with sufficient available material were screened for the presence of the HPV genome, which was detected in the DNA of two tumours (14%) (Figure 3). Both cases were carcinomas with squamous metaplasia.

Discussion

The number of cases of pure SCC in the literature is small and the largest series have described only 22 cases in 1990 (22) and 33 cases in another study published recently (23).

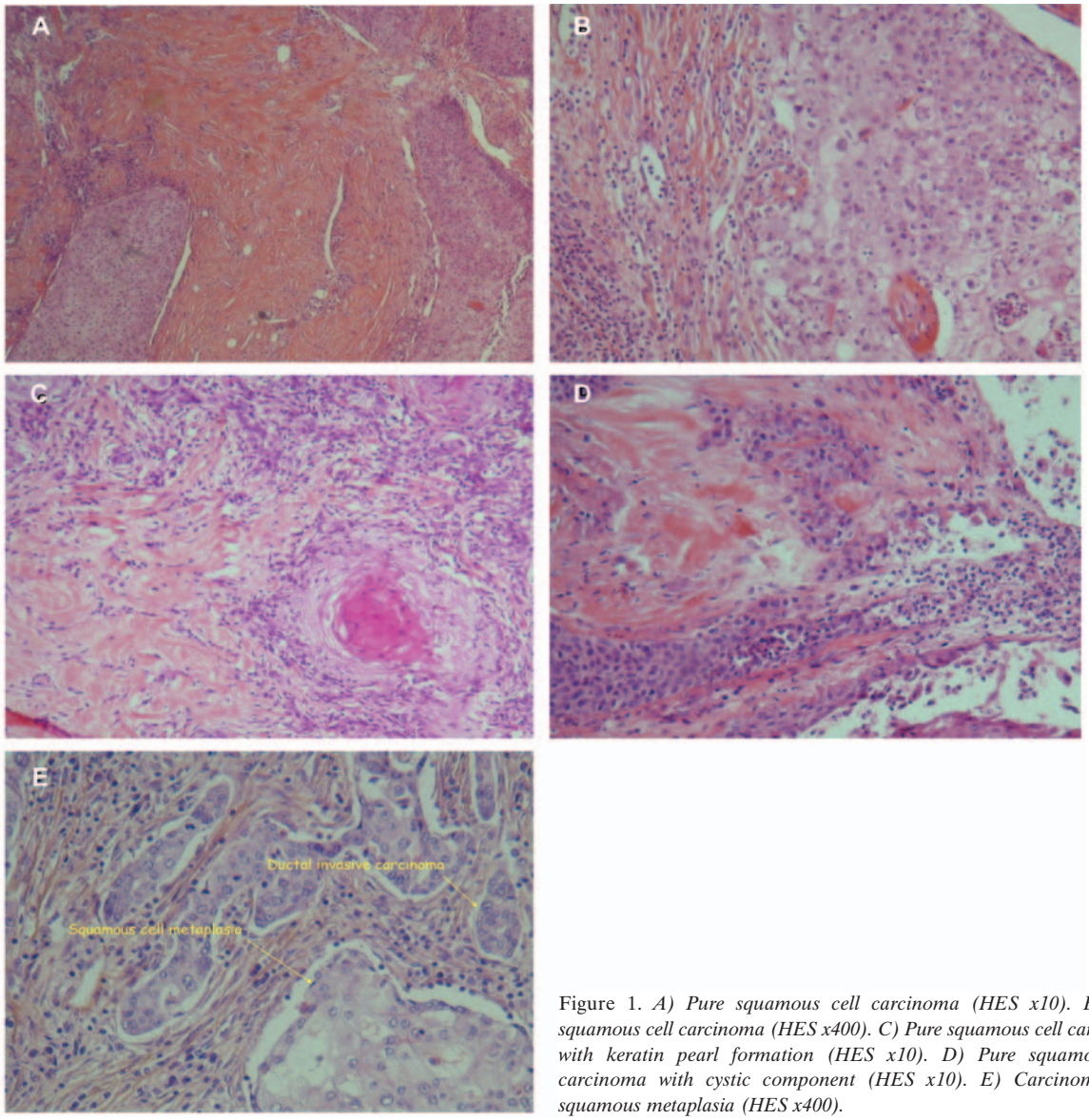


Figure 1. *A) Pure squamous cell carcinoma (HES x10). B) Pure squamous cell carcinoma (HES x400). C) Pure squamous cell carcinoma with keratin pearl formation (HES x10). D) Pure squamous cell carcinoma with cystic component (HES x10). E) Carcinoma with squamous metaplasia (HES x400).*

The specific oncogenic pathways which give rise to this unusual pathological subtype of breast cancer remain unknown. The usual risk factors for the development of SCC in various organs, such as smoking or alcohol consumption, do not seem to play a role in the onset of SCC of the breast. Due to a potential viral etiology, the possibility of an HPV infection has been investigated, but it was found that it is not a leading event in the oncogenesis of breast SCC. Indeed the 14% prevalence of HPV infection which was found in our series was consistent with the prevalence of HPV described in ductal invasive carcinoma in the literature (24-26).

Various pathophysiological hypotheses have been put forward to explain the development of SCC of the breast. Most authors have suggested that it originates from squamous metaplasia (10, 27-30). Such metaplasia has been observed in the epithelium of cysts, fibroadenomas, phyllode tumours, duct hyperplasia and papillomas (31). It has also been found in inflammatory lesions, such as chronic abscesses. Some authors have suggested that squamous metaplasia originates from myopepithelial cells (12). Finally, an alternative postulation has proposed that this entity may have an embryologic (ectodermic) origin. An attempt to describe the specific immunohistochemical profile of this cancer has therefore been undertaken in the hope of identifying the cell of origin and possibly the oncogenic pathway.

There is currently a growing body of evidence for the clinical and therapeutic value of the molecular profile

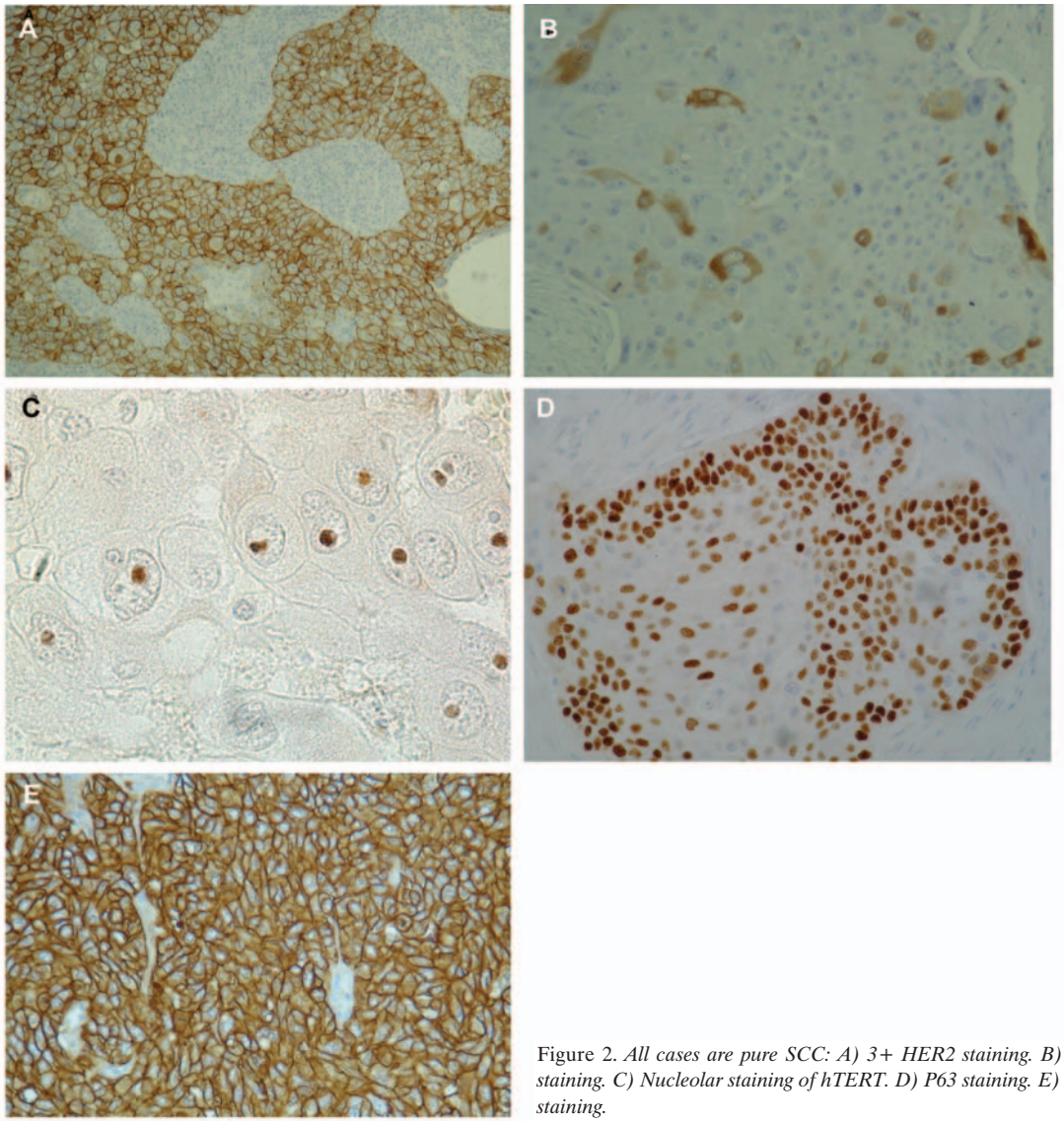


Figure 2. *All cases are pure SCC: A) 3+ HER2 staining. B) Cyclin B1 staining. C) Nucleolar staining of hTERT. D) P63 staining. E) 3+ EGFR staining.*

subclassification of breast carcinoma, although in reality it remains controversial. The molecular entities described have been associated with quite different outcomes. Basal-like carcinomas have been repeatedly shown to be aggressive tumours with a poor outcome (32-34). They are the subtype observed in BRCA1-related breast cancer (16). Specific profiles have also been purported to have differential sensitivities to chemotherapy (35). It is not clear whether the so-called basal-like carcinoma has a pure basal or a basal/myoepithelial origin (36, 37). Some authors have suggested that p63 may be a specific marker of a myoepithelial origin while others have found that it may reflect both a myoepithelial and a basal origin.

In this study we have shown that tumours that were morphologically described as SCC of the breast had a profile that was typical of the so-called basal/myoepithelial phenotype. Indeed, they were "triple-negative" (ER-, PR- and HER2-negative); in more than 90% of the cases, they overexpressed either EGFR or CK5/6 in 85% and 75%, and p63 in 70% of the cases, and were highly proliferative (high expression of Ki-67). It is noteworthy that this profile has been demonstrated in more than 95% of SCC, irrespective of whether they were pure or metaplastic tumours.

As other tumours previously described as basal-like (pure or metaplastic) squamous cell carcinomas of the breast have long been associated with a poor prognosis and relative resistance to conventional therapy targeting the breast, several teams have also considered "basal-like" breast cancers to be potentially resistant to conventional anthracycline-based therapies and have proposed the development of specific targeted therapies such as Her1-directed antagonists or monoclonal antibodies (38) for the management of this subtype. SCC of the breast

Table IV. *Expression of ER, PR, Ki-67, EGFR, HER2, Cyclin B1, hTERT, CK 5/6 and p63 in SCC and controls.*

	Squamous cell carcinoma	Invasive ductal carcinoma	*p*
N	27	27	
ER			
Positive	0	20 (80%)	<0.0001
Negative	27 (100%)	5 (20%)	
PR			
Positive	0	16 (64%)	<0.0001
Negative	27 (100%)	9 (36%)	
Ki-67			
>20%	21 (77%)	7 (28%)	0.017
<20%	5 (23%)	15 (72%)	
EGF-R			
>10%	23 (85%)	3 (12%)	0.0041
<10%	4 (15%)	22 (88%)	
HER2			
3+	2 (7%)	2 (8%)	0.93
0-2+	25 (93%)	23 (92%)	
Cyclin B1			
>5%	16 (60%)	4 (16%)	0.0001
<5%	11 (40%)	21 (84%)	
hTERT			
>10%	16 (60%)	16 (64%)	0.53
<10%	11 (40%)	9 (36%)	
CK 5/6			
Positive	18 (75%)	0 (0%)	0.0006
Negative	6 (25%)	18 (100%)	
p63			
Positive	12 (70%)	2 (11%)	0.11
Negative	5 (30%)	16 (89%)	

Table V. *Expression of ER, PR, Ki-67, EGFR, HER2, Cyclin B1, hTERT, CK 5/6 and p63 in pure versus metaplastic carcinomas.*

	Pure squamous	Metaplastic squamous	*p*
N	11	16	
ER			
Positive	0	0	ns
Negative	11 (100%)	16 (100%)	
PR			
Positive	0	0	ns
Negative	11 (100%)	16 (100%)	
Ki-67			
>20%	8 (72%)	13 (81%)	0.37
<20%	3 (27%)	2 (19%)	
EGF-R			
>10%	9 (81%)	14 (87%)	0.68
<10%	2 (19%)	2 (13%)	
HER2			
3+	1 (9%)	1 (6%)	0.78
0-2+	10 (91%)	15 (94%)	
Cyclin B1			
>5%	5 (45%)	10 (62%)	0.38
<5%	6 (55%)	6 (38%)	
hTERT			
>10%	6 (55%)	10 (62%)	0.67
<10%	5 (45%)	6 (38%)	
CK 5/6			
Positive	7 (63%)	10 (62%)	0.27
Negative	1 (9%)	5 (31%)	
p63			
Positive	5 (42%)	7 (58%)	ns
Negative	0	5 (42%)	

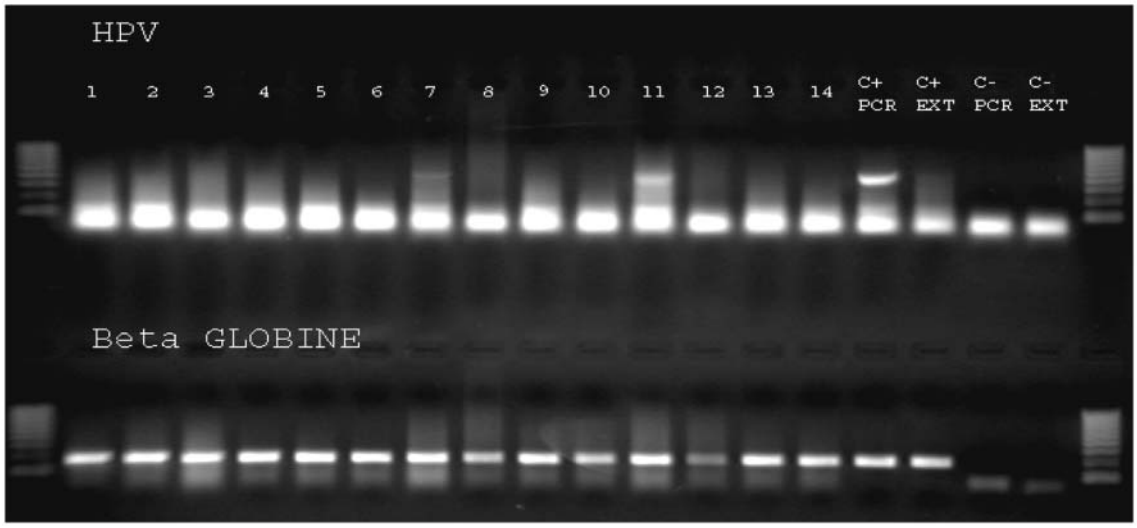
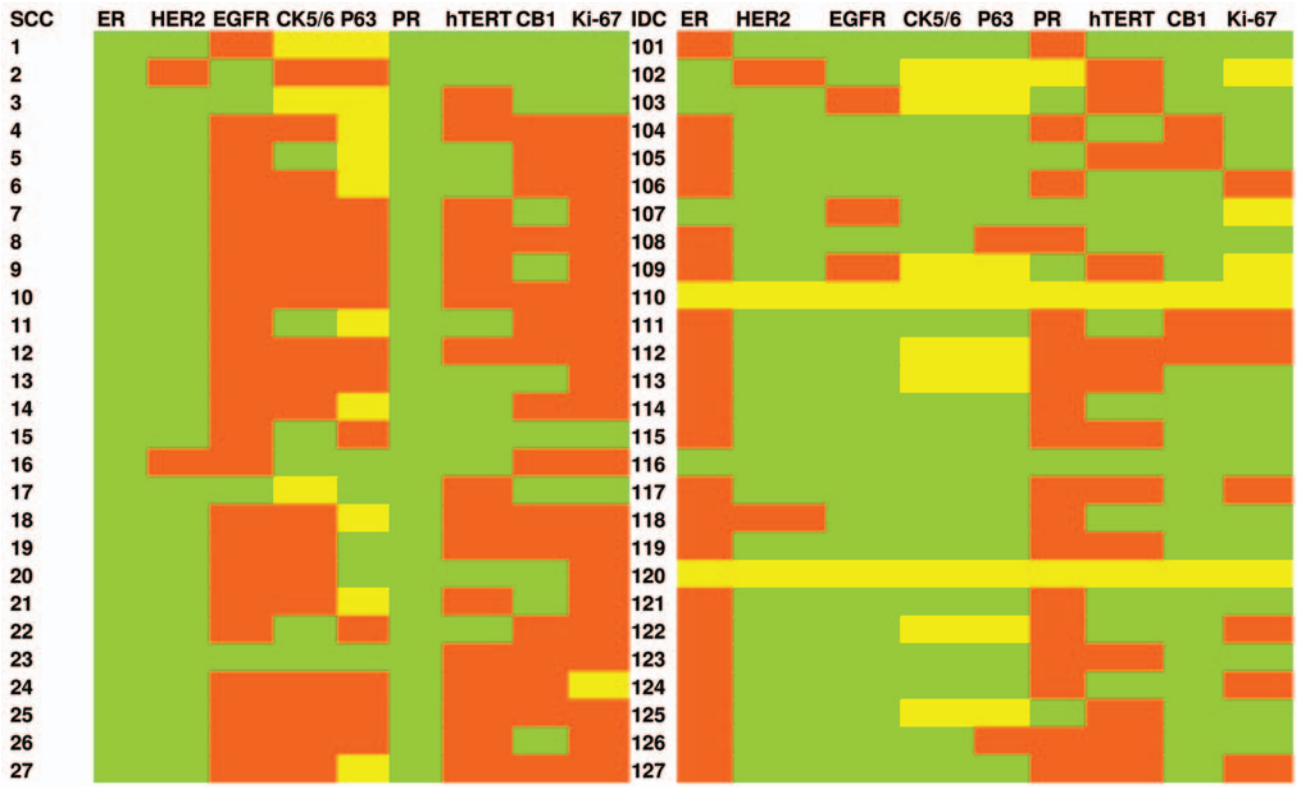


Figure 3. *HPV PCR: positive cases are numbers 7 and 11.*

therefore appears to be a paradigm for such potentially chemoresistant disease and a candidate for specific targeted therapies, as it has been repeatedly described as resistant to some conventional therapies (23). In this study, the dismal prognosis associated with this entity and therefore the need for specific targeted therapy has been confirmed.

Table VI. *Immunohistochemical profile of SCC and control breast cancers. CB1: Cyclin B1. In Red: positive case. In Green: negative case. In Yellow: not done or not evaluable.*



Other breast cancer subtypes have recently been described as belonging to the newly-defined group of breast cancer molecular subtypes. For instance, it has been suggested that medullary carcinomas (39), and also very recently, adenoid cystic carcinomas, could be described as members of the "basal-like" family (40, 41). This begs the question whether the molecular subclassification of tumours may be a more relevant way of distinguishing cancers with a similar clinical behaviour and therapeutic needs than the conventional morphological description.

To complete analysis, it would have been interesting to evaluate the expression profile of BRCA1 protein in our series. For instance, the BRCA1 expression was only found in 26% of sporadic breast cancer (ductal invasive breast cancer) in a recent study (42) and no correlation was observed with HER1 or HER2. Since SCC display a basaloid phenotype and usually show poor prognostic features, we expect to find a lack of BRCA1 expression.

In conclusion, squamous cell carcinoma of the breast displayed a phenotype typical of the recently characterised subgroup of "basal-like" breast cancer. This may explain a large part of their aggressive clinical behaviour and resistance to conventional therapies, and may signify that dedicated therapeutic approaches are warranted for these tumours.

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