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Report

IPH-926 lobular breast cancer cells are triple-negative but their microarray profile uncovers a luminal subtype

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Human primary breast cancers and breast cancer cell lines are classified by microarray-defined molecular subtypes, which reflect differentiation characteristics. Estrogen receptor (ER) expression is indicative of the luminal molecular subtype. We have previously established IPH-926, the first well–characterized cell line from infiltrating lobular breast cancer. IPH-926 displays an ER/PR/ErbB2 triple-negative immunophenotype, which is due to a loss of ER expression in its *in vivo* clonal ancestry. Loss of ER might indicate a fundamental change of cellular differentiation and it is unclear whether a luminal subtype is preserved beyond ER conversion. Using Affymetrix microarray analysis, seven different classifier gene lists (PAM305, DISC256, TN1288, PAM50, UNC1300, LAB704, INT500) and a background population of 50 common mammary carcinoma cell lines, we have now determined the molecular subtype of IPH-926. Strikingly, the IPH-926 expression profile is highly consistent with a luminal subtype. It is nearest to luminal/ER-positive breast cancer cell lines and far apart from basal breast cancer cell lines. Quantitative real–time RT–PCR confirmed enhanced expression of luminal marker genes (*AGR2*, *CLU*, *CA12*, *EMP2*, *CLDN3*) and low or absent expression of basal marker genes (*KRT5*, *CD44*, *CAV1*, *VIM*). Moreover, IPH-926 lacked androgen receptor (AR) expression, a transcription factor previously associated with luminal-like gene expression in a subset of triple-negative or molecular apocrine breast cancers. In conclusion, IPH-926 is triple-negative but belongs to the luminal subtype. Luminal differentiation characteristics can be preserved beyond ER conversion and might not require a compensatory expression of AR. (*Cancer Sci* 2013; 104: 1726–1730)

Microarray expression analyses identified breast cancer molecular subtypes, such as luminal, basal-like and ErbB2-positive carcinomas, which share differentiation characteristics.[1–4] This molecular taxonomy is continually refined.[5] However, for clinical diagnostics, molecular subtypes are approximated by immunohistochemical surrogate markers.[6] The 2011 and 2013 St Gallen consensus conferences have confirmed immunohistochemistry for estrogen receptor (ER), progesterone receptor (PR) and ErbB2 as the clinical gold standard to assess tumor phenotypes corresponding to microarray-defined molecular subtypes.[7,8] However, this has not been without dissenting votes, as these immunohistochemical markers are primarily directed towards finding the optimal targeted therapy and show only a limited concordance with microarray-defined molecular subtypes.[8] For instance, an ER/PR/ErbB2 triple-negative (TN) immunophenotype is typically seen in carcinomas classified as basal-like by microarray analysis, but only 30–70% of TN carcinomas are basal, as defined by gene expression profiling.[9,10] Accordingly, basal and TN phenotypes must not be equated.[9]

Human breast cancer cell lines are also classified by microarray-defined subtypes.[11–14] This is relevant for pre-clinical models.[15] The PAM305 classifier gene list has been established specifically for subtype assignment in breast cancer cell lines.[11] Like primary tumors, cell lines might show a luminal subtype (typically ER-positive) or a basal subtype (typically TN).[11,12] The basal subtype is commonly stratified into groups termed basal-A and basal-B.[11,12] An alternative subdivision for TN breast cancer has been described by Lehmann *et al.*[10] and includes subclasses termed basal-1 and basal-2 (BL1/BL2), immunomodulatory (IM), mesenchymal and mesenchymal stem–like (M/MSL) and luminal androgen receptor (AR)-positive (LAR). Contrary to primary tumors, the luminal subtype is not further subdivided and ErbB2–positive samples do not form a class of their own in breast cancer cell lines.[11,12]

Infiltrating lobular breast cancer (ILBC) is a special histological entity associated with ER expression and the luminal subtype.[16,17] IPH-926 is the first well–characterized cell line from ILBC.[18–20] We have previously shown that IPH-926 displays a TN immunophenotype, which is due to a progression-related loss of ER in its *in vivo* clonal ancestry.[18] Estrogen receptor conversion is observed in approximately 10% of initially ER-positive carcinomas and might indicate a loss of luminal differentiation.[21] Hence, the molecular subtype of IPH-926 is enigmatic. Here we report the first comprehensive microarray-based classification of IPH-926, which adds important new information as to the exact categorization of this unique cell line and the stability of luminal differentiation.

Materials and Methods

Cell lines. IPH-926 cells were authenticated by short tandem repeat profiling and PCR–based detection of the unique *CDH1* 241ins4 mutation.[18]

Immunohistochemistry. Immunohistochemical stainings were performed on a Benchmark Ultra (Ventana, Tucson, AZ, USA) automated stainer using the CC1 mild protocol for antigen retrieval and the monoclonal anti-ER (clone SP1, undiluted read-to-use; Ventana), anti-PR (clone 1E2, undiluted read-to-use; Ventana), anti-ErbB2 (clone 4B5, undiluted read-to-use; Ventana) and anti-AR (clone AR441, 1:40; Dako, Glostrup, Denmark) antibodies.

Microarray analyses. Affymetrix U133Plus2.0 (Santa Clara, CA, USA) GeneChip raw data of IPH-926 (GEO GSE28089)[20]

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and 50 common breast cancer cell lines (GEO GSE12777)[14] were combined and analyzed using Expression Console and BRB-array tools software as outlined in the Data S1.

Quantitative real-time RT-PCR. Quantitative assessment of gene expression normalized to the housekeeping gene *GUSB* was performed with Platinum Taq DNA polymerase (Invitrogen, Darmstadt, Germany), Sybr Green I (Invitrogen) and QuantiTect primer assays (Qiagen, Hilden, Germany) on an ABI Prism 7700 system (Applied Biosystems, Foster City, CA, USA).

Results

The IPH-926 ILBC cells displayed a TN immunophenotype (Fig. 1a).[18] To assess the molecular subtype of IPH-926, we analyzed the Affymetrix U133Plus2.0 microarray expression data of IPH-926 (internal data set) on a background population of 50 common breast cancer cell lines (external data set) (Fig. 1b). Sufficient comparability of internal data and external expression data was confirmed by evaluating two cell lines (MCF-7, BT-474) provided in the external data and profiled at our own laboratory, which showed near perfect whole array signal log values (SLV) correlation ($r_s > 0.95$, not shown). Hierarchical clustering of the combined data set based on the PAM305 classifier gene list reproduced distinct clusters of cell lines (basal-A, basal-B, luminal) as described previously.[11,12] IPH-926 was included in such an analysis for the first time and was part of the luminal cluster (Fig. 1c).

Hierarchical clustering is not reliable for molecular subtype assignment.[3] A more objective approach is to test for a Spearman correlation $r_s > 0.1$ of the test sample's expression profile with the mean expression profiles (e.g. centroids) of prototypic samples representative for each subtype.[3,17] Thus, PAM305 centroids were computed for the basal-A, basal-B and the luminal subtype based on prototypic cell lines highlighted in several independent studies.[11–14] Prototypic cell lines selected for the luminal subtype included ER-positive/ErbB2-negative cell lines only. Prototypic cell lines selected for the basal subtypes included TN cell lines only. Then, Spearman correlation of the IPH-926 expression profile with the three PAM305 class centroids was determined. IPH-926 was nearest to the PAM305 luminal centroid ($r_s = +0.51$) and was far apart from the basal-A ($r_s = -0.17$) and basal-B ($r_s = -0.50$) centroids, indicating a bona fide luminal subtype (Fig. 1d).

Next, the IPH-926 microarray profile was validated using quantitative real-time RT-PCR. For this purpose, $n = 10$ marker genes were chosen. *AGR2, CLU*, *CA12*, *EMP2* and *CLDN3* were included based on their high SLV in the IPH-926 microarray profile (Fig. 1d, upper circle). These genes are established luminal markers.[11,12] *KRT5, CD44, CAV1* and *VIM* were included based on their low SLV in the IPH-926 microarray profile (Fig. 1d, lower circle). These genes are preferentially expressed in the basal-A (*KRT5, CD44*) or basal-B

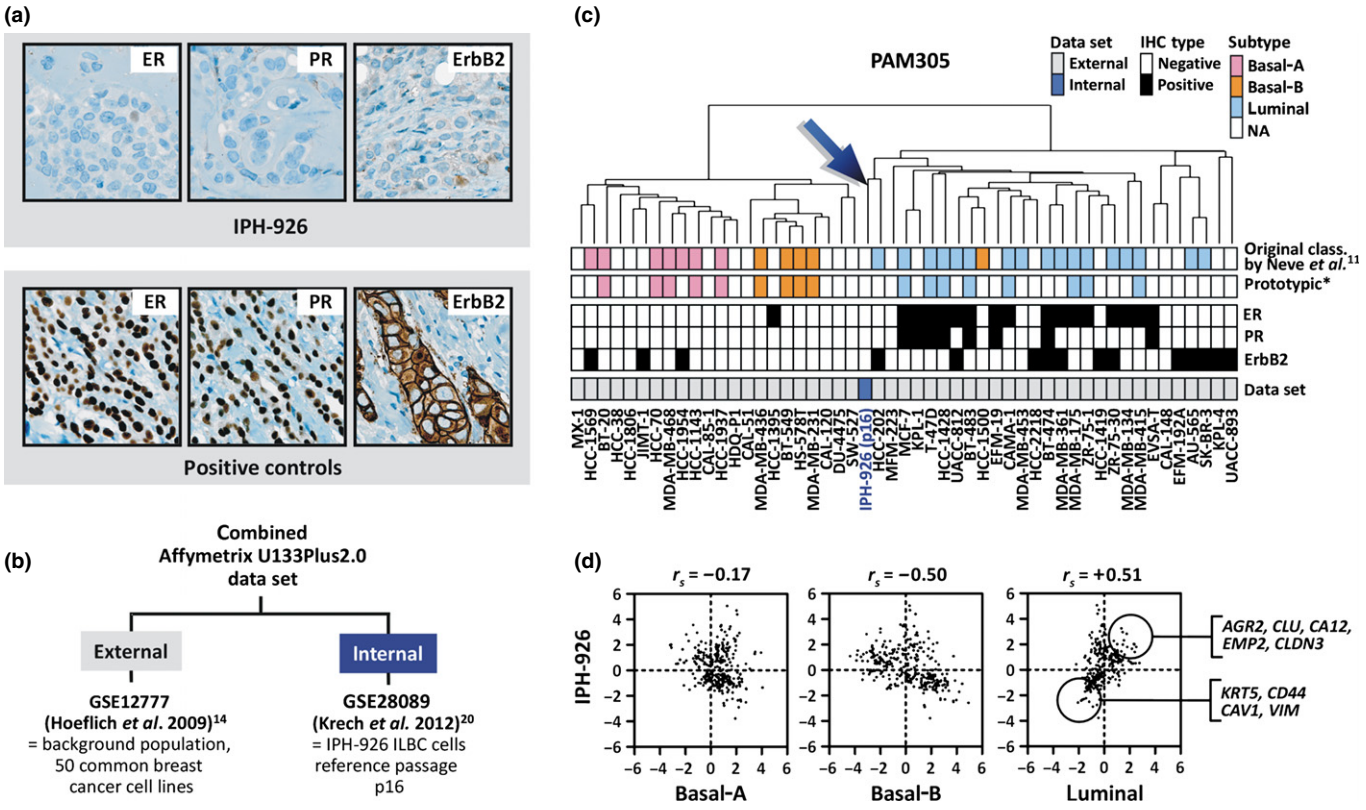

Fig. 1. (a) Immunohistochemical staining for estrogen receptor (ER), progesterone receptor (PR) and ErbB2. (b) Overview on the external and internal microarray data sets and their Gene Expression Omnibus (GEO) accession numbers.[14,20] (c) Hierarchical clustering of 51 human breast cancer cell lines using the PAM305 classifier gene list.[11] IPH-926 (p16) refers to the reference passage p16. *Cell lines considered as prototypic based on their repeated synonymous classification in independent studies[11–14] are highlighted. The ER, PR and ErbB2 status was retrieved from the literature[11–14,18]. NA, not assessed/not classified in previous studies.[11] (d) Spearman correlation of the IPH-926 expression profile with the PAM305 basal-A, basal-B and luminal centroids based on prototypic cell lines computed from the external data set. Each dot represents a single probe set. Marker genes for quantitative real-time RT–PCR validation were chosen from the numerous probe sets with high signal log values (SLV) in IPH-926 and the luminal centroid (upper circle) or low SLV in IPH-926 and the luminal class centroid (lower circle).

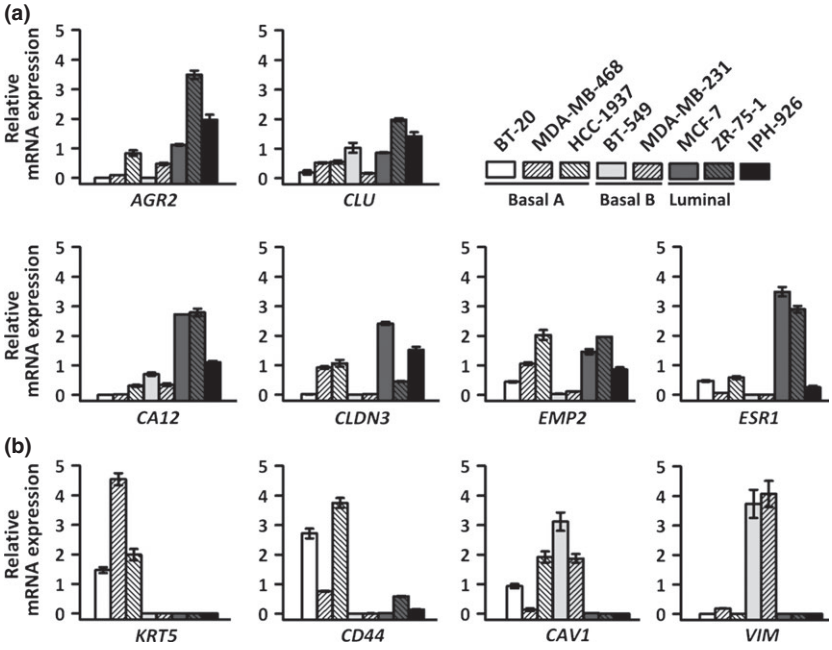

Fig. 2. (a) Validation of the IPH-926 microarray profile using quantitative real–time RT–PCR for luminal marker genes. (b) Quantitative real–time RT–PCR for basal-A/basal-B marker genes. Data are presented as relative mRNA expression with the mean of each gene's expression across the eight cell lines analyzed adjusted to a value of 1 for better visualization. Error bars indicate SEM calculated from three independent experiments.

Table 1. Spearman correlation of the IPH-926 profile with the centroids of prototypic basal-A, basal-B and luminal cell lines

Classifier gene list	Original reference	Established for classification of	Original platform	Representation on Affymetrix U133Plus2.0	Cluster of cell lines that contains IPH-926	IPH-926 [r_s]		
						Basal-A	Basal-B	Luminal
PAM305	Neve *et al.*[11]	Breast cancer cell lines	Affymetrix	305 probe sets	Luminal	−0.17	−0.50	+0.51
DISC256	Guedj *et al.*[5]	Primary breast cancers	Affymetrix	375 probe sets	Luminal	−0.16	−0.37	+0.27
TN1288	Lehmann *et al.*[10]	Primary breast cancers and cell lines	Cross-platform compilation	5213 probe sets	Luminal	−0.03	−0.21	+0.19
PAM50	Parker *et al.*[4]	Primary breast cancers	qRT-PCR	132 probe set	Separate	−0.16	−0.21	+0.03
UNC1300	Hu *et al.*[3]	Primary breast cancers	Agilent	2925 probe set	Luminal	−0.07	−0.13	+0.19
LAB704	Farmer *et al.*[23]	Primary breast cancers	Affymetrix	902 probe sets	Luminal	−0.18	−0.29	+0.16
INT500	Sorlie *et al.*[2]	Primary breast cancers	Stanford cDNA array	1309 probe sets	Luminal	−0.05	−0.15	+0.20

(*CAV1*, *VIM*) subtype.[11,12] *ESR1*, encoding for ER, was also included, although we have previously shown that IPH-926 cells express little or no *ESR1* mRNA.[18] Quantitative real-time RT-PCR was carried out for IPH-926, three prototypic basal-A cell lines, two prototypic basal-B cell lines and two prototypic luminal cell lines (Fig. 2). Consistent with the microarray data, IPH-926 showed enhanced expression of all luminal marker genes, except *ESR1* (Fig. 2a), and low or absent expression of basal-A/basal-B marker genes (Fig. 2b). Hence, quantitative real-time RT-PCR confirmed the validity of the IPH-926 microarray profile.

The PAM305 classifier gene list was devised for breast cancer cell lines.[11] Other classifier gene lists have been established for primary breast cancers.[2–5] Altered *in vitro* growth and the lack of tumor stroma are reasonable grounds to suppose that classifiers optimized for primary tumors should not be used for cell lines and *vice versa*.[11] In fact, studies using laser capture microdissection have suggested that subtype assignment with some classifiers is at least partially dependent on the specific gene expression characteristics of the stroma associated with different types of tumors.[22] However, as this is a matter of debate, we repeated all analyses for the INT500, LAB704, UNC1300, PAM50, TN1288 and DISC256 classifiers, which were all optimized for primary tumors. Hierarchical clustering based on these alternative classifiers reproduced essentially the same clusters of cell lines (basal-A, basal-B, luminal) as seen with the PAM305 classifier (Fig. S1). Spearman correlation of the IPH-926 profile with the centroids of prototypic basal-A, basal-B and luminal cell lines showed that IPH-926 was always nearest to the luminal centroid, irrespective of the classifier gene list (Table 1). This additionally corroborated that IPH-926 cells belong to the luminal molecular subtype.

Androgen receptor (AR) induces a luminal-like transcriptional program in some TN breast cancers.[24] A new molecular subclass, termed luminal AR-positive (LAR), has been established to cover this phenotype, which overlaps with the molecular apocrine subtype.[10,23] Based on the TN1288 classifier gene list, Lehman *et al.*[10] have defined prototypic LAR-type breast cancer cell lines, such as TN/AR-positive MDA-MB-453 cells. To assess whether the gene expression of IPH-926 reflects a LAR-type cellular differentiation, the combined microarray data set was reanalyzed (Fig. 3). Similar to the approach of Lehmann *et al.,* only TN cell lines were retained for a refined hierarchical clustering based on the TN1288 classifier gene list. As a result of this, IPH-926 clustered together with LAR-type cell lines (Fig. 3a). The IPH-926 profile was positively correlated with the centroid of LAR-type

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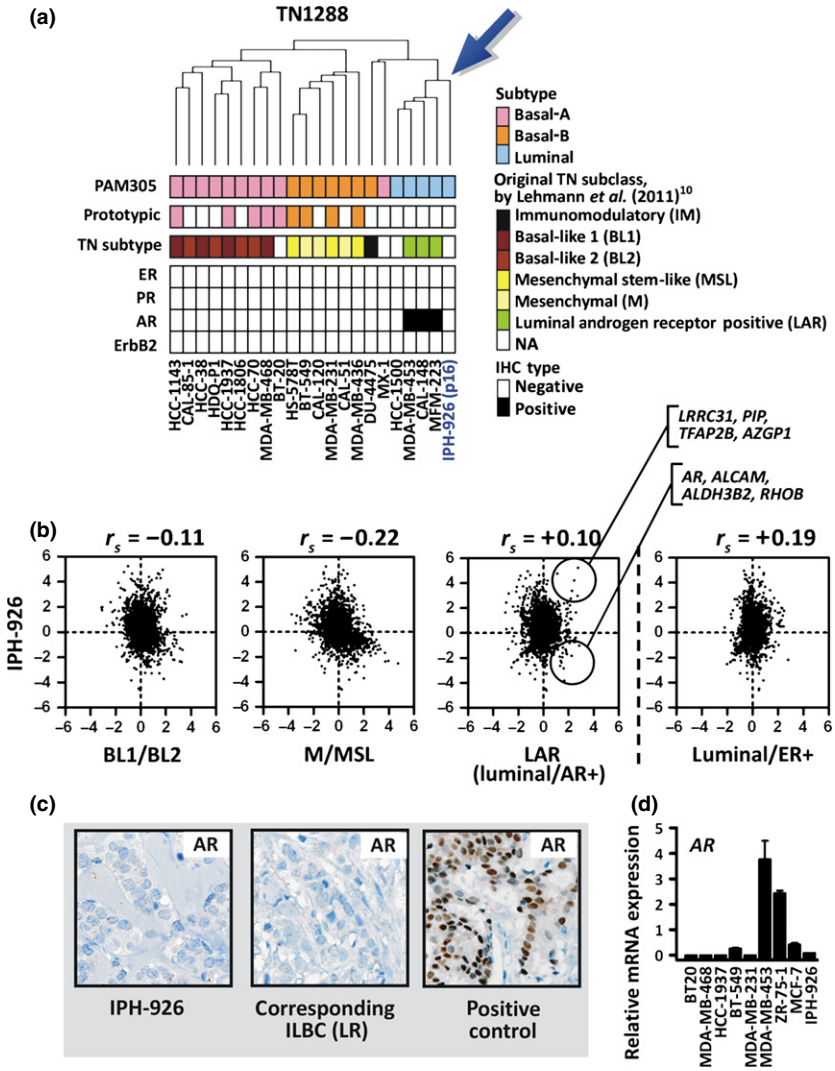

Fig. 3. (a) Hierarchical clustering of 22 human triple-negative (TN) breast cancer cell lines using the TN1288 classifier gene list.[10] The estrogen receptor (ER), progesterone receptor (PR), ErbB2 and androgen receptor (AR) status was retrieved from the literature.[10–14, 18] Please note that the immunophenotype of HCC-1500 cells is controversial.[10–12] (b) Spearman correlation of the IPH-926 expression profile with the TN1288 BL1/BL2, M/MSL and LAR (luminal/AR+) centroids based on prototypic TN breast cancer cell lines computed from the external data set. Spearman correlation of the IPH-926 expression profile with the TN1288 luminal/ER-positive centroid was also calculated (right plot). Each dot represents a single probe set. The upper circle highlights marker genes with high signal log values (SLV) in the LAR subclass and IPH-926. The lower circle highlights marker genes with low SLV in IPH-926 but high SLV in the LAR subclass. (c) Immunohistochemical staining for AR. The middle panel shows the original locally recurrent (LR) lobular carcinoma corresponding to IPH-926. (d) Quantitative real–time RT–PCR for *AR*. Data are presented as relative mRNA expression with the mean across the nine cell lines tested adjusted to a value of 1. Error bars indicate SEM calculated from three independent experiments.

cell lines, but it was nearest to the centroid of prototypic luminal/ER-positive cell lines, which were excluded from the clustering analysis (Fig. 3b). In line with the microarray data, IPH-926 cells were AR-negative, as determined using immunohistochemistry and quantitative real-time RT-PCR, suggesting that IPH-926 is not a LAR-type breast cancer cell line (Fig. 3c,d).

Discussion

IPH-926 is the first well-characterized ILBC cell line and displays a TN immunophenotype.[18–20] This is due to a progression-related loss of ER in its *in vivo* clonal ancestry.[18] More specifically, the corresponding patient had experienced ER conversion in the locally recurrent lobular carcinoma corresponding to the IPH-926 cell line.[18] Loss of ER might indicate a fundamental change of cellular differentiation. Accordingly, the molecular subtype of IPH-926 has remained undefined. Determining the molecular subtype of IPH-926 cells is important for two reasons: (i) IPH-926 is increasingly used as a pre–clinical model, urging for its categorization in relation to other breast cancer cell lines; and (ii) IPH-926 may provide evidence that luminal differentiation can be preserved beyond ER conversion.

Here we report that the IPH-926 microarray expression profile is consistent with a luminal subtype. It was nearest to luminal/ER-positive breast cancer cell lines and far apart from basal breast cancer cell lines. This was independent from the classifier gene list, arguing for the robustness of this categorization. Quantitative real-time RT-PCR confirmed that IPH-926 cells express luminal marker genes, such as *AGR2*, but lack basal marker genes, such as *KRT5* or *VIM*. Unfortunately, a direct comparison of microarray profiles before and after loss of ER was impossible because of unavailability of cryoconserved tissue of the early, still ER-positive ILBC corresponding to the IPH-926 cell line.[18]

Recent studies have drawn attention to TN breast cancers with luminal gene expression characteristics. The AR has emerged as an important driver of luminal differentiation in TN breast cancer and the molecular apocrine and LAR breast cancer subtypes were established to cover this phenotype.[10,23,24] Among breast cancer cell lines, TN/AR-positive MDA–MB–453 cells were first described as luminal breast cancer cells, but were later defined more precisely as LAR-type breast cancer cells.[10] IPH-926 cells are AR-negative and their gene expression profile is more closely related to prototypic luminal/ER-positive than to prototypic luminal/AR-positive representatives, suggesting that IPH-926 is not a LAR-type breast cancer cell line.

In conclusion, IPH-926 is best classified as a luminal breast cancer cell line, similar to MCF-7, despite its TN immunophenotype. This will impact on the future use of IPH-926 in breast cancer research. Moreover, our finding implies that a luminal expression profile is not simply erased if its prime clinical surrogate marker, ER, is lost. Maintenance of a luminal differenti-

ation following to ER conversion might not even require compensatory expression of AR. This may help to further improve our understanding of TN mammary carcinomas with otherwise luminal gene expression.

Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Hierarchical clustering of 51 human breast cancer cell lines using the INT500, LAB704, UNC1300, PAM50, TN1288 and DISC256 classifier gene list.[2–5]

Data S1. Including: microarray data analyses; and classifier gene lists.

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