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Intermediate conductance Ca^{2+} activated K^+ channels are expressed and functional in breast adenocarcinomas: correlation with tumour grade and metastasis status

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Summary. K^+ channels are key molecules in the progression of several cancer types and are considered to be potential targets for cancer therapy.

In this study, we investigated the intermediate-conductance Ca^{2+}-activated K^+ channels (hKCa3.1) expression in both breast carcinoma (BC) specimens and human breast cancer epithelial primary cell cultures (hBCE) using immuno-histochemistry (60 samples), quantitative Real-Time RT-PCR (30 samples) and Western blot assay (30 samples). We also looked at whether or not the expression of these channels is correlated with breast carcinomas grade tumours and metastasis status. Furthermore, we characterized the hKCa3.1 channel activity in hBCE cells by using the Whole Cell Patch Clamp Technique.

We found that hKCa3.1 transcripts and proteins were expressed in both BC samples and hBCE cells. Clinicopathologic evaluation indicated a significant correlation between hKCa3.1-expression and tumour grade. hKCa3.1 mRNA and protein were more highly expressed in grade III tumours than in both grades I and II. However, the hKCa3.1 expression-increase according to grade was only observed in tumours with negative metastasis status. Moreover, the hKCa3.1 channels expressed in hBCE cells are functional. This was attested by patch-clamp recordings showing typical hKCa3.1-mediated currents in these cells. In conclusion, these data suggest that hKCa3.1 might contribute to breast tumour-progression and can serve as a useful prognostic marker for breast cancer.

Key words: Intermediate conductance Ca^{2+}-activated K^+ channels, Human breast cancer epithelial primary culture, Tumour grade, Metastatic status

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Introduction

Dysfunction and/or the expression of potassium (K^+) channels contribute to the appearance of many pathologies, including carcinogenesis (Pardo et al., 2005). Indeed, K^+ channels have been suggested as tumour markers and therapeutic targets for cancers, including breast cancer (Schonherr, 2005; Pardo and Suhmer, 2008).

hKCa3.1 is a Ca^{2+} activated intermediate conductance K^+ channel with little or no voltage dependency (Nehrke et al., 2003; Begenisich et al., 2004). hKCa3.1 channels are found in red blood cells and Lymphocytes (Jensen et al., 1999; Hoffman et al., 2003; Maher and Kuchel, 2003). Many epithelial cells express hKCa3.1 (Ishii et al., 1997; Warth et al., 1999; Thompson-Vest et al., 2006). Pharmacological hKCa3.1 inhibition by the two known potent and selective hKCa3.1 blockers (clotrimazole and its analogues TRAM-34), or specific down-regulation by siRNA against hKCa3.1 suppress the proliferation of several cancer cells *in vitro*, such as prostate cancer cells (Parihar et al., 2003), pancreas (Jager et al., 2004), endometrial (Wang et al., 2007), breast (Ouadid-Ahidouch et al., 2004), and lymphoma cells (Wang et al., 2007). All these studies of the role of hKCa3.1 were

carried out on the cell lines but not on native cancer cells. hKCa3.1 expression was also found in some cancer tissues. Indeed, the mRNA level for hKCa3.1 is up-regulated in pancreatic cancer tissue (Jager et al., 2004) and human endometrial cancer tissues (Wang et al., 2007), suggesting a possible role for the channel in oncological diseases.

Using PCR and immunohistochemistry techniques, we and others have reported that several K^+ channel types are expressed in breast cancer tissues (Stringer et al., 2001; Mu et al., 2003; Brevet et al., 2008). Breast tumour tissues express voltage-dependant K^+ channels (Kv1.1, Kv1.3), Ca^{2+}-activated large conductance K^+ channels (BK_{Ca}), G-protein inwardly rectifying potassium channel 1 (GIRK1) (Stringer et al., 2001) and the two-pore domain (TWIK) related to acid-sensitive (TASK) channels (Mu et al., 2003). Both the expression of GIRK1 and BK_{Ca} was positively correlated with lymph-node metastasis and estrogen receptors respectively (Stringer et al., 2001; Brevet et al., 2008).

Although hKCa3.1 channels in MCF-7 cells have been studied in great detail by our group (Ouadid-Ahidouch and Ahidouch, 2008), very little is known about this channel conductance in human native breast cancer cells and their expression in human breast adenocarcinomas. Therefore, we characterized the hKCa3.1 channels in hBCE, and we looked into whether these channels are expressed in primary invasive breast carcinomas, and then correlated the results with breast carcinomas from negative (LNM^-) and positive (LNM^+) auxillary lymph nodes and grade tumours.

We show, for the first time, that hKCa3.1 are functional in hBCE. Moreover, the expression of these channels is positively correlated with high grade tumour and LNM^-.

Materials and methods

Primary culture

Portions of human breast cancerous tissues were enzymatically dissociated as previously reported (Guilbert et al., 2008). Each sample was analyzed by immuno-fluorescence staining to verify the pan-cytokeratin expression, which is an epithelial marker. The absence of normal epithelial cells was confirmed by independent histologic and anatomopathologic analysis.

Electrophysiology

For electro-physiological analysis, cells were cultured in 35 mm Petri dishes 2 days before patch clamp experiments. Currents were recorded in voltage-clamp mode, using an Axopatch 200 B patch-clamp amplifier (Molecular devices) and a Digidata 1200 interface (Molecular device). PClamp software (v. 6.03, Molecular device) was used to control voltage, as well as to acquire and analyze data. The whole-cell mode of the patch-clamp technique was used with 3-5 MΩ resistance borosilicate fire-polished pipettes (Hirschmann®, Laborgerate). Seal resistance was typically in the 1-5 GΩ range. Whole cell currents were allowed to stabilize for 5 min before being measured. Cells were allowed to settle in Petri dishes placed at the opening of a 250 μm-inner diameter capillary for extra-cellular perfusions. The cell under investigation was continuously superfused with control or test solutions. All electrophysiological experiments were performed at room temperature.

Solutions

External and internal solutions had the following compositions (in mM): External: NaCl 140, KCl 5, $MgCl_2$ 2, $CaCl_2$ 2, HEPES 10 and glucose 5 at pH 7.4 (NaOH). Internal: KCl 150, HEPES 10, EGTA 0.5, $MgCl_2$ 2, at pH 7.2 (KOH). In order to completely block the voltage activated K^+ channels, we added TEA at 2 mM to the extracellular medium. TRAM-34, clotrimazole, and Iomomycin (Sigma, France) were dissolved in DMSO. Final concentrations were obtained by appropriate dilution in an external control solution. The final DMSO concentration was <0.1%.

Immunohistochemistry

Cancerous breast tissues were obtained from fresh surgical specimens. Surgical consent forms (approved by the University Hospital of Amiens) were signed by the patients before surgery, to allow the use of a portion of the tissue for research purposes. The Scarff, Bloom and Richardson histopronostic grade established a rank of tumours in 3 separate grades (I, II, III). Immuno-histochemical studies were performed as previously described (Guilbert et al., 2008) using the indirect immuno-peroxidase staining technique on paraffin-embedded material with a Ventana ES automatic analyzer (Ventana Medical Systems) and with a hematoxylin counterstain. After staining with an anti-hKCa3.1 antibody (Santa Cruz, 1/300), the slides were examined under an optical microscope.

Immuno-staining levels in the tumour tissue were determined by subjective visual scoring of the brown stain. Scoring levels were: 1: weak staining intensity; 2: moderate; 3: strong staining intensity. For the quantitative analysis, we report the percentage of cases presenting a low expression of hKCa3.1 (score 1) and high expression of hKCa3.1 (scores 2 and 3).

Qualitative PCR

Total RNA from MCF-7 cells, primary culture cells and pieces of tissues were extracted as previously detailed (Guilbert et al., 2008). Sense and anti-sense PCR primers specific to hKCa3.1 channels (QT00224112, Qiagen), ß-actin (5'-CAGAGCAAG AGAGGCATCCT-3' and 5'-ACGTACATGGCTG GGGTG-3') (Eurobio) were used. Qualitative PCR

reactions were carried out on an iCycler thermal cycler (Biorad), using Taq DNA polymerase (Invitrogen) using the following parameters: denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 40 s. A total of 23 cycles for actin and 30 cycles for the hKCa3.1 primers were performed, followed by a final extension at 72°C for 7 min. PCR products were analyzed by electrophoresis with 2% agarose gel and visualized by ethidium bromide staining.

Real-time reverse transcriptase-PCR analysis

Total RNA was treated with amplification-grade DNase I (Invitrogen). First-strand cDNA was synthesized using oligodT primers and MultiScribeTM Reverse Transcriptase (Applied Biosystems). These cDNAs were used for PCR experiments using sense and antisense PCR primers specific to hKCa3.1 channels (QT00224112, Qiagen) and cytokeratin 19 (CK19) (5'-GATTGCCACCTACCGC-3' and 5'-CCATCCCT CTACCCAG-3') (Eurobio) were used as gene-specific primers. All primers were optimized for use with an annealing temperature of 60°C during PCR. Real-time PCR was performed on a Roche LightCycler® using the ABsolute™ QPCR SYBR® Green Capillary Mix (ABgene®) in a final volume of 20 μl according to the manufacturer's protocol.

All reactions were prepared in duplicate and performed twice. Crossing-point values, which are the PCR cycle numbers at which the accumulated fluorescent signal in each reaction crosses a threshold above background, were obtained with the LightCycler® software 3.5 (Roche), using the second derivative maximum method. Crossing-point values are a function of the amplification efficiency of the respective PCR. These data were then exported into the RelQuant© software (Roche). This software provides efficiency-corrected, calibrator-normalized quantification results. Results are expressed as the target/reference ratio of the sample. The efficiency-corrected quantification performed by RelQuant© is based on relative standard curves describing the PCR efficiencies of each target and the reference gene.

Relative standard curves are determined and used for each analysis. For each gene of interest, the same melting point and size were observed for PCR products obtained by real time RT-PCR using cDNAs, thus indicating a low probability of false priming.

Western blotting

MCF-7 cells and primary culture cells were lysed for 30 min on ice in RIPA buffer (1% triton X100, 1% Na deoxycholate, 150 mM NaCl, 10 mM PO_4Na_2/K pH 7.2) supplemented with Sigma P8340 inhibitor cocktail, 2 mM EDTA and 5 mM Na orthovanadate. After centrifugation, the proteins in the supernatant were quantified using the BCA method (Biorad).

Breast tissue proteins were extracted using the WCE buffer (Whole Cell Extract : 150 mM NaCl, 50 mM Tris HCl pH7.5, 1% NP40) supplemented with Sigma P8340 inhibitors cocktail, 0.1% SDS and 1 mM Na orthovanadate. After 1 h in lysis buffer at 4°C, tissues were homogenized using a Polytron homogenizer (PRO-200, Fisher Bioblock Scientific) and frozen for 20 min at -80°C. After centrifugation, the proteins in the supernatant were quantified using the BCA method (Biorad).

Equal amounts of each protein sample were separated by electrophoresis on SDS-PAGE and blotted onto nitrocellulose membrane (Amersham). Blots were incubated with antibodies raised against hKCa3.1 (1/1000, Santa Cruz) or ß-actin (1/1000, Santa Cruz) and developed with the enhanced chemi-luminescence system (ECL, Amersham) using specific peroxidase-conjugated anti-IgG secondary antibodies.

Statistical analysis

Data are presented as mean ± S.E.M. Statistical differences of data were evaluated by Student's t-test. χ^2 tests were used in GraphPad Software to estimate the correlation between hKCa3.1 expression and clinical characteristics of the carcinoma tissues. A correlation was considered significant when $p<0.05$.

Results

hKCa3.1 are expressed in hBCE cells and mediate currents

Both hKCa3.1 transcripts (Fig. 1A) and protein were found in hBCE cells (Fig. 1B). MCF-7 and HEK stably transfected by hKCa3.1 (HEK-hKCa3.1) were used as positive controls (Fig.1A-B). We then investigated whether the hKCa3.1 were functional. Under whole-cell recording conditions, the membrane potential (MP) measured 5 min after accessing the intracellular compartment was -9.2±5.2 mV (n=15) and the outward currents elicited with ramp voltage from -80 to +80 mV for 350 ms showed a strongly voltage-dependent K^+ current (Fig. 1C). This voltage-dependent current was largely inhibited by 2 mM TEA, a well-known K^+ channel blocker (Fig. 1C), suggesting that the predominant outward current recorded in hBCE was a voltage-activated K^+ channel. Thus, to characterize the properties of hKCa3.1 channels in hBCE, we performed experiments in the presence of 2 mM TEA in the bath to prevent any potential contamination by the voltage-dependent current. Under these conditions, the MP varied between -20 mV and 11 mV with a mean of 0.25±2.2 mV (n=25) and little or no outward current was activated. Extracellular perfusion of 1 μM ionomycin induced a hyperpolarization of the MP (-45.9±4.1 mV (n=20)) and activated an outward current which was characterized with a small inward component (Fig. 1D-E). The reversal potential was around -80 mV (-82±0.9 mV, n=15). Then, the cell currents were

pharmacologically characterized with the two known hKCa3.1 blockers (i.e. clotrimazole and TRAM-34). Voltage ramps (-120 to 100 mV, 300 ms duration) were applied every 20 s. The 5 μM clotrimazole perfusion essentially blocked a linear component without affecting the reversal potential (Fig. 1D) and depolarized the MP to -3.3±1.2 (n=8). Moreover, the current ionomycin-sensitive was also blocked by TRAM-34 (5 μM, n=10, Fig. 1E) and also depolarized the MP (-5.2±1.7, n=6). Additionally, in HEK-hKCa3.1 cells, extracellular perfusion of ionomycin (1 μM) activated a linear current and hyperpolarised the MP (-60.8±3.2 mV, n=13). The current ionomycin-sensitive was inhibited both by TRAM-34 and clotrimazole (Fig. 1 F-G).

hKCa3.1 are also expressed in breast adenocarcinoma

hKCa3.1 proteins were first shown on 60 breast cancer tissue samples by using immuno-histochemistry. hKCa3.1 expression was demonstrated in 100% of the cases studied. Figure 2A shows a representative positive hKCa3.1 expression in breast adenocarcinoma tissue (Fig. 2A-a). There was little or not staining when the primary antibody was omitted (Fig. 2A-b). Using PCR

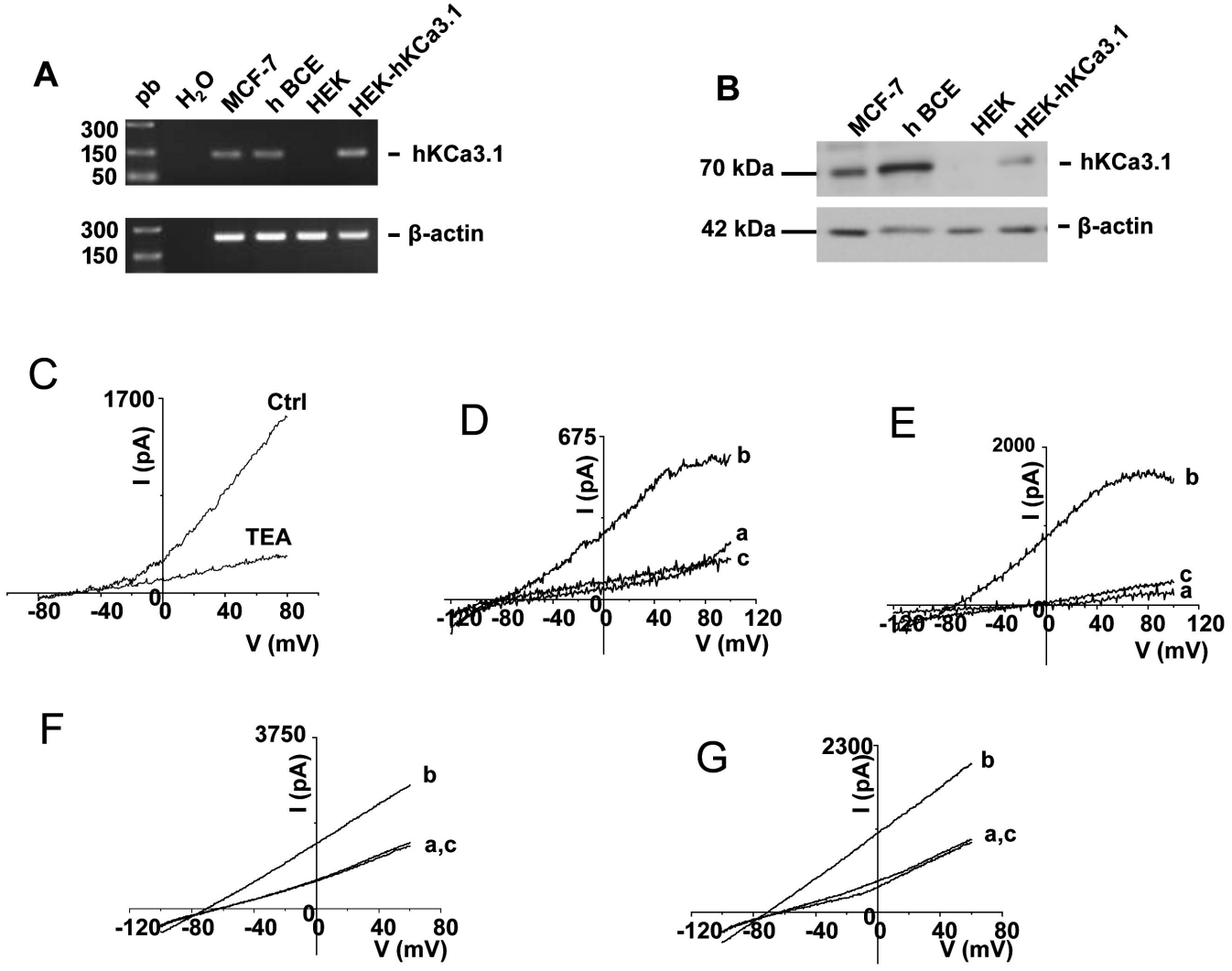

Fig. 1. hKCa3.1 is expressed in the primary epithelial culture and is functional. **A.** Expression pattern of hKCa3.1 mRNA in hBCE. MCF-7 and HEK-hKCa3.1 cells were used as positive controls and HEK cells as a negative control. **B.** A representative Western blot of hKCa3.1 performed on hBCE, MCF-7 and HEK-hKCa3.1. **C.** Typical current trace recorded in hBCE cells in the control conditions and after extracellular perfusion of TEA (2 mM). The holding potential was -40 mV and 350 ms voltage ramps were applied from -80 mV to +80 mV. **D.** (a) K^+ current recorded in control conditions (2 mM TEA), perfusion of ionomycin (1 μM) activated K^+ current (b), which is completely inhibited by Clotrimazole (5 μM, c). **E.** TRAM 34 (5 μM, c) inhibited the K^+ current induced by ionomycin (1 μM, b). **F-G.** Effects of TRAM-34 (5 μM, c) and Clotrimzole (5 μM, c) on the K^+ current induced by ionomycin (1 μM, b) in HEK-hKCa3.1 cells respectively.

technique, we found an expression of hKCa3.1 transcripts in all the neoplastic breast tissue samples tested. Both HEK-hKCa3.1 and MCF-7 cells were used as a positive control (Fig. 2B). Again, when using Western blotting, we found that hKCa3.1 proteins were expressed in breast carcinoma (Fig. 2C).

hKCa3.1 expression correlated with the tumour grade and lymph node metastasis

We investigated whether the expression of hKCa3.1 was correlated with tumour grade and metastasis status. hKCa3.1 mRNA and protein were evaluated by Q-PCR and Western blot in 33 cases where frozen tissues were available and immunohistochemical tests were carried out on 60 samples. Regarding tumour grade in the 33 invasive ductal breast carcinomas, 10 were of Grade I, 12 were of Grade II and 11 were of Grade III. On diagnosis, 17 tumours presented lymph-node metastasis (LNM^+) and 16 tumours did not (LNM^-). On comparison with the tumour grade, the hKCa3.1 mRNA level was higher in grade III tumours than in grades I and II (Fig.

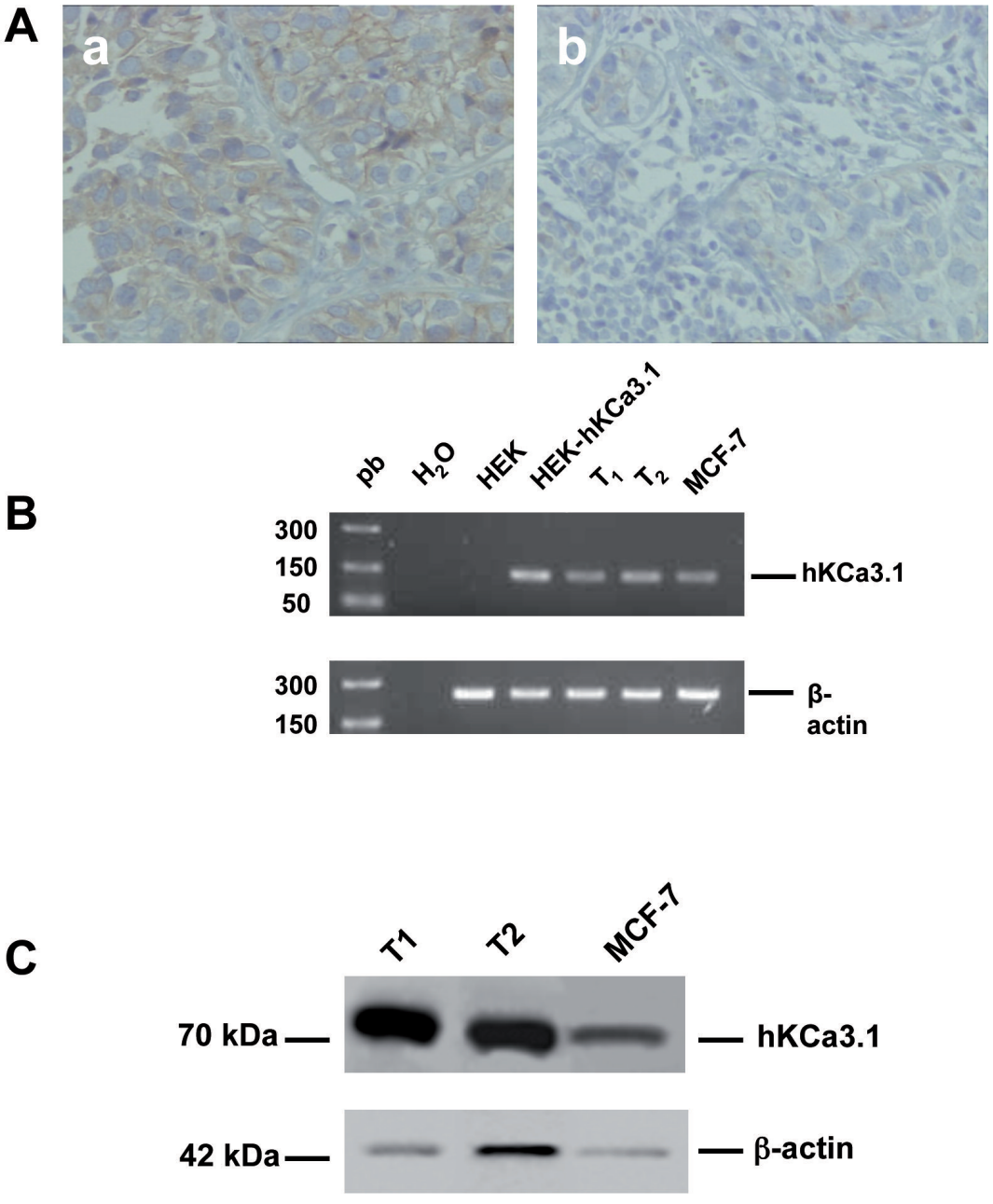

Fig. 2. hKCa3.1 expression in breast adenocarcinoma. **A.** Immunostaining of hKCa3.1 in breast cancer tissues presented staining when incubated with an hKCa3.1 antibody (a), and no staining when the primary antibody was omitted (b). x 200. **B.** hKCa3.1 transcripts were detected in HEK-hKCa3.1, MCF-7 cells and in the breast tissues (T1, T2) by qualitative PCR. **C.** A representative Western blot of hKCa3.1 was performed on breast tumour tissues (T1, T2).

3A). In contrast, hKCa3.1 transcripts decreased in tumours associated with metastasis (Fig. 3B). We next investigated the expression of hKCa3.1 channels in tumour grades I, II and III, whether associated or not with metastasis. Figure 3C shows that hKCa3.1 mRNA levels increased only with tumour grades LNM^-. Indeed, in tumours LNM^-, the quantity of transcripts is 5 times higher in grade III than in grade I (Fig. 3C) and 2.6 fold between grade II and III (Fig. 3C). Furthermore, when comparing hKCa3.1 transcripts in tumour grades not associated with metastasis to those that are, we showed a decrease by 2.9 and 5 fold in grade II and III tumours respectively (Fig. 3C). However, no differences are visible when comparing the expression of hKCa3.1 between tumour grades LNM^+ (Fig. 3C). Using Western blot, we find that the amount of hKCa3.1 protein increased only in grade III in LNM^- tumours (Fig. 3D). When comparing grades I and III LNM^- with LNM^+, we found that hKCa3.1 protein increased by 2.3 and 3.2 fold respectively (Fig. 3D).

A quantitative analysis, using a χ^2 statistical test, of the results obtained is reported in Table 1. In tumours LNM^-, we found a high hKCa3.1 immunostaining in 75% and 63.6% of grades I and II respectively. This immunostaining increased to reach 100% in grade III. Figure 4 shows a representative hKCa3.1 immuno-

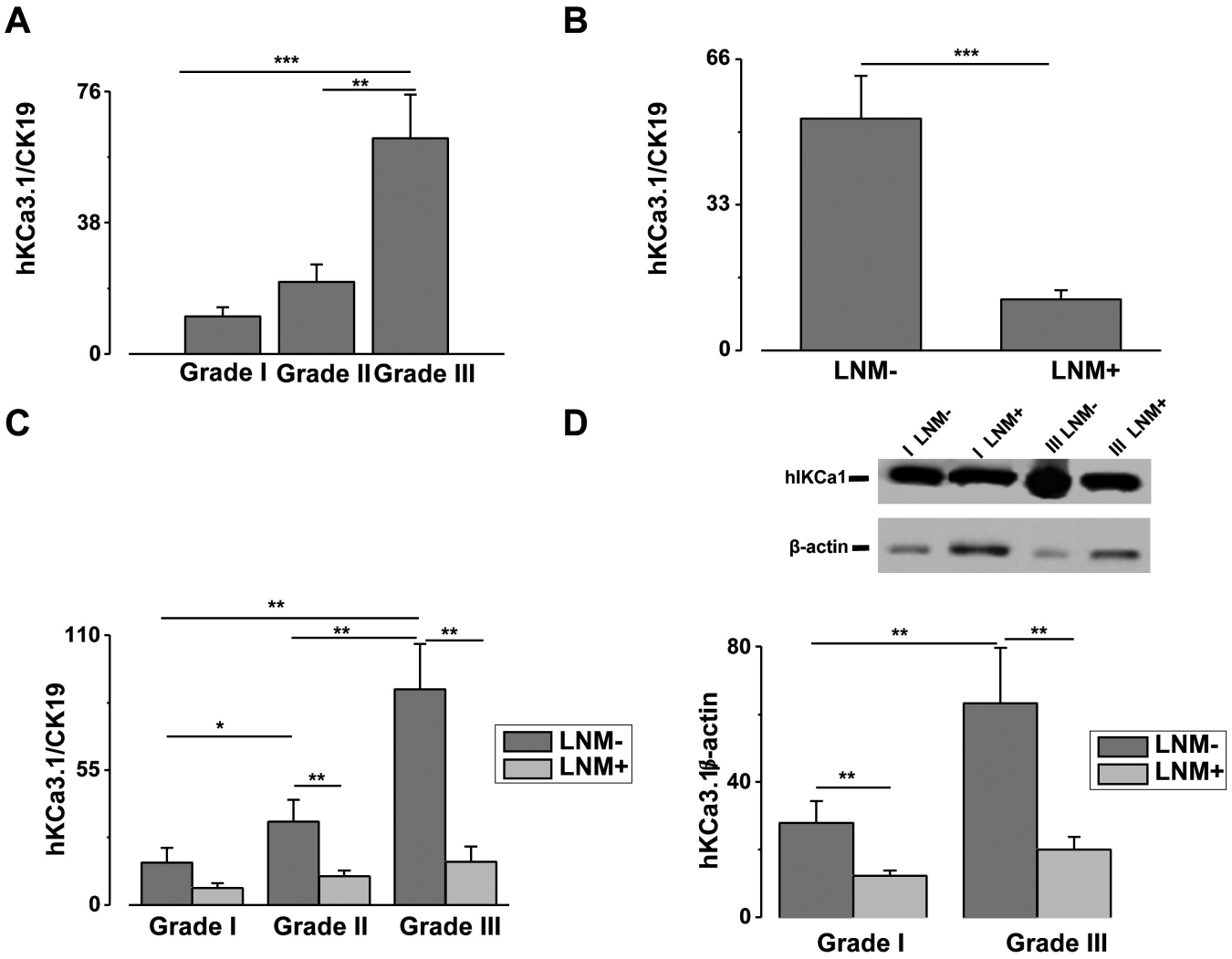

Fig. 3. Correlation between hKCa3.1 expression and grade and lymph node metastasis status. Expression of hKCa3.1 in breast tumour correlated with tumour grade **(A)**, and with metastatic status **(B)**; (LNM^-) without metastasis, (LNM^+) with metastasis. ***: p<0.001, **: p<0.01. **C.** Expression of hKCa3.1 in tumour grades I, II and III LNM^+ or LNM^-. *: p< 0.05, **: p< 0.01. **D.** Protein expression of hKCa3.1 performed on grades I and III LNM^+ or LNM^-. Top panels are representative immunoblots and bottom panels are mean densitometric data from 33 individual experiments normalized to ß-actin. **: p< 0.01, *: p<0.05.

histochemistry observed in tumour grades LNM^+ (Fig. 4A-C) or LNM^- (Fig. 4D-F).

We also investigated the expression of hKCa3.1 in lymph nodes positive for metastatic carcinoma at different grades. Our data showed that very little hKCa3.1 are expressed in the lymph nodes (Fig. 5).

Table 1. Comparison of hKCa3.1 expression to tumour characteristics on 60 patients using $\chi 2$ analysis.

	LNM^-				LNM^+			
Histological grade	Low	High	n	$\chi 2$	Low	High	n	$\chi 2$
I	25%	75%	8		22.2%	77%	9	
II	36.4%	63.6%	11*	0.04	0%	100%	4	0.57
III	0%	100%	15**	0.01	46.1%	46.1%	13	0.09

*: hKCa3.1 expression in grade III/grade II; **: hKCa3.1 expression in grade III/grade I.

Discussion

hKCa3.1 channels have been found in the MCF-7 cell line and are involved in controlling the cell cycle, and therefore cell proliferation. However, no previous reports have described hKCa3.1 channel expression in native primary breast cancer cells.

In hBCE cells, we identified both mRNA and protein encoding the hKCa3.1 channels, and demonstrated the plasma membrane expression of functional channels using patch clamp electrophysiology. Moreover, the expression of hKCa3.1 is correlated with tumour grade and metastasis status.

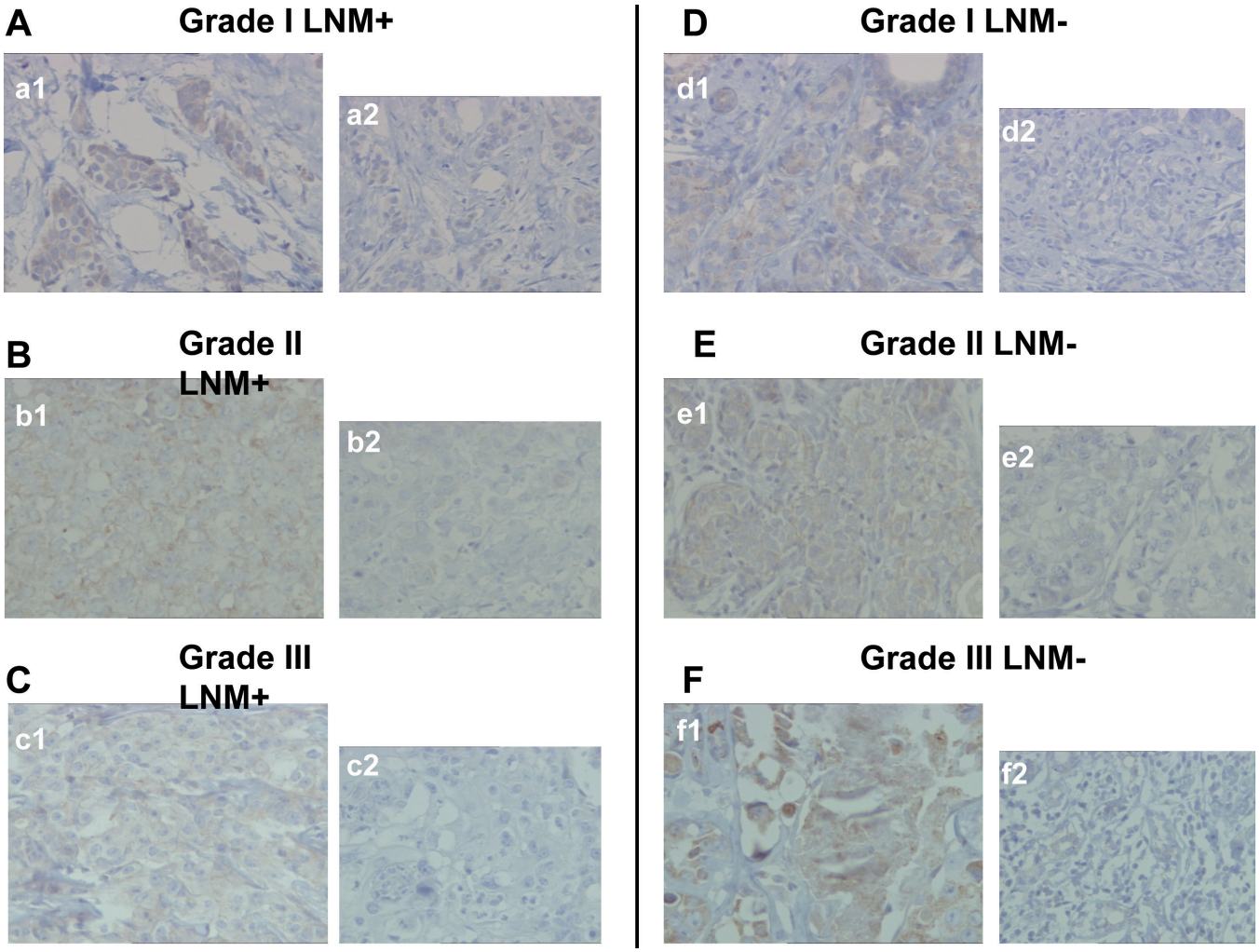

Fig. 4. Representative hKCa3.1 immunochemistry in tumour grades whether associated or not with metastasis. Tumours associated **(A-C)** and not with metastasis **(D-F)**. (a1-f1) Breast tumour tissues showed staining when incubated with hKCa3.1 antibody, (a2-f2) are the same field showing no staining when the primary antibody was omitted. x 200

Tumour cells typically showed a depolarized resting membrane potential (MP). The mean MP in hBCE was depolarized compared with that measured in similar conditions in the MCF-7 cell line (Ouadid-Ahidouch et al., 2001). In contrast, this value was close to the MP measured by standard microelectrodes in breast cancer tissues (Marino et al., 1994). Moreover, as shown in MCF-7 cells (Ouadid-Ahidouch et al., 2004), hKCa3.1 in hBCE cells controls the membrane potential by inducing a hyperpolarization.

A very important question is whether there is a correlation between the disease stage and hKCa3.1 expression. Our study was done on ductal human breast cancer specimens, the most frequent pathology (80%, (Miron et al., 2008). hKCa3.1 were detectable in all the breast tumours tested. However, although the hKCa3.1 immunostaining signal was cytoplasmic, our electrophysiological measurements showed active hKCa3.1 in hBCE cells. To our knowledge, only 2 studies have demonstrated an over-expression of hKCa3.1 in tumour tissue, i.e. in pancreatic (Jager et al., 2004), and in endometrial cancer tissues (Wang et al., 2007). However, they did not study the hKCa3.1 variations in clinical parameters. Our results show clearly that hKCa3.1 are expressed at high steady state levels in advanced breast carcinoma not associated with metastasis suggesting their involvement more in the breast development than in migration and/or invasion. Indeed, Wang et al., have suggested the involvement of hKCa3.1 in the development of endometrial cancer, since the injection of the endometrial cancer cell line HEC-1-A, which overexpresses hKCa3.1, into nude mice induced the formation of tumours, and that the inhibition of hKCa3.1 by clotrimazole and TRAM-34 slowed tumour growth.

In the human breast cancer cell line MCF-7, our team (Ouadid-Ahidouch et al., 2004; Ouadid-Ahidouch and Ahidouch, 2008) has reported that hKCa3.1 regulates cell proliferation by inducing a membrane hyperpolarization, which in turn acts as an essential requirement for inducing cell progression in the G1 phase and G1/S transition. Moreover, solid tumours are also characterised by oxygen deprivation which could affect the channel activity. Indeed, Tajima et al. have reported in the melanoma cell line that the activity of hKCa3.1 was significantly enhanced by low oxygen conditions. Taken together, hKCa3.1 expression and/or

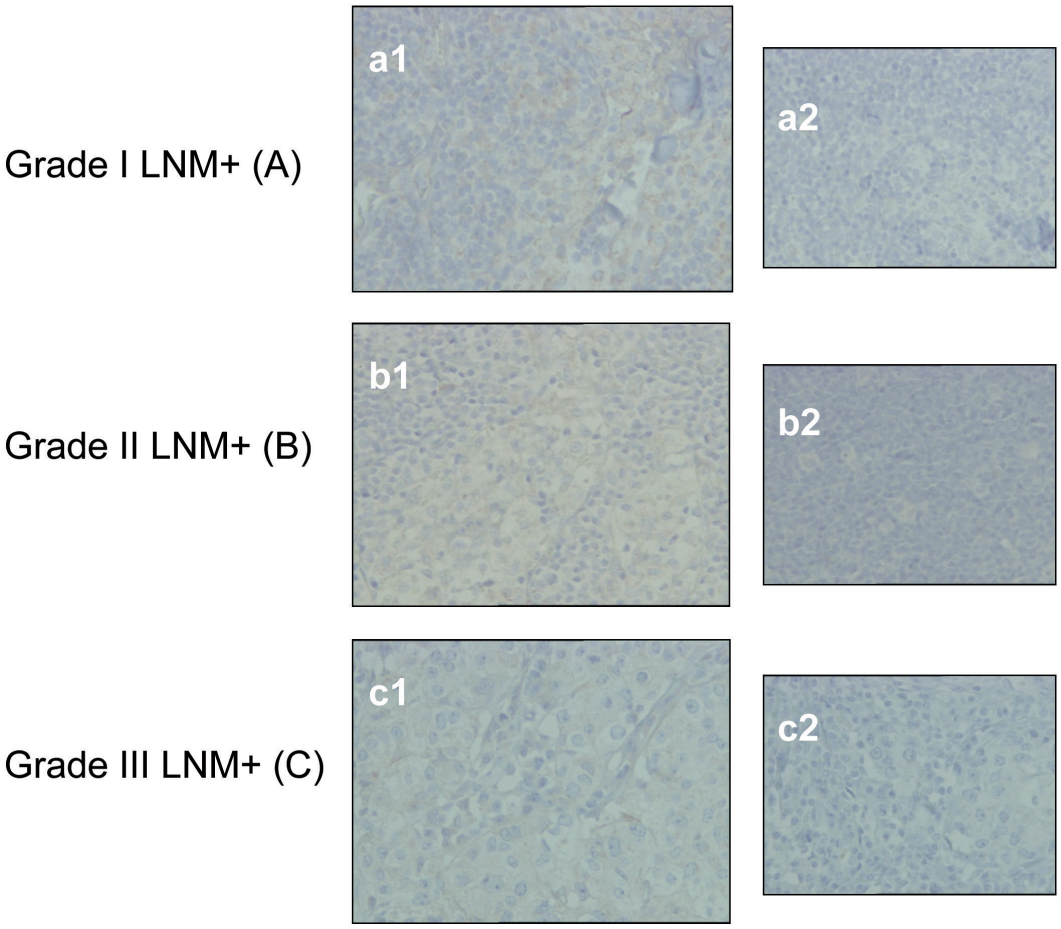

Fig. 5. Representative hKCa3.1 immunochemistry in lymph nodes associated with metastasis and grades. **A.** Grade I LNM+. **B.** grade II LNM+. **C.** grade III LNM+. (a1-c1) breast tumour tissues showed slight staining when incubated with hKCa3.1 antibody, (a2-c2) the same fields showing no staining when the primary antibody was omitted. x 200

activity may confer a growth advantage to tumour cells and permit a selective enrichment of hKCa3.1-expressing cells.

Because hKCa3.1 channels are involved in the regulation of breast cell proliferation, and their expression increases with the higher tumour grades, we can suggest the use of these channels as a potential new proliferation marker in breast cancer.

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