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herg1 Gene and HERG1 Protein Are Overexpressed in Colorectal Cancers and Regulate Cell Invasion of Tumor Cells

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ABSTRACT

The acquisition of the capacity to invade surrounding tissues confers a more malignant phenotype to tumor cells and is necessary for the establishment of metastases. The understanding of the molecular mechanisms underlying cell invasion in human solid tumors such as colorectal cancers could provide not only more sensitive prognostic analyses but also novel molecular targets for cancer therapy.

We report in this article that K^+ ion channels belonging to the HERG family are important determinants for the acquisition of an invasive phenotype in colorectal cancers. The *herg1* gene and HERG1 protein are expressed in many colon cancer cell lines, and the activity of HERG channels modulates colon cancer cell invasiveness. Moreover, the amount of HERG1 protein expressed on the plasma membrane is directly related to the invasive phenotype of colon cancer cells.

Finally, both the *herg1* gene and HERG1 protein were expressed in a high percentage of primary human colorectal cancers, with the highest incidence occurring in metastatic cancers, whereas no expression could be detected either in normal colonic mucosa or in adenomas.

INTRODUCTION

Cancer development is a multistep process, which includes not only alterations in cell growth and survival, but also the acquisition of the ability to invade surrounding tissues (1, 2). The final step of tumor progression is the establishment of metastases, which represent the major hindrance to cancer therapy (1, 2).

It has been proposed that the altered setting of the plasma membrane electric potential (V_{rest}) of cancer cells can contribute to tumor growth (3). Our studies have shown that V_{rest} in tumor cells can often be modulated by the activity of K^+ channels, encoded by the *human ether a-gò-gò related gene* (*herg*; Ref. 4). The corresponding current, I_{HERG}, can contribute to driving the V_{rest} of tumor cells to more depolarized values, due to the peculiar biophysical properties of I_{HERG} (5). Both the *herg* gene and HERG protein are indeed expressed in tumor cell lines of differing histogenesis (4), as well as in primary human cancers, such as endometrial adenocarcinomas (6), acute myeloid leukemias (7), and lymphoid leukemias (8). In leukemia (7, 8) and neuroblastoma cells (5, 9), HERG channel activity modulates the progression through the mitotic cycle; in these cells an alternative transcript of the *herg1* gene, *herg1b*, was cloned and shown to be mainly expressed during the S phase of the cell cycle (9). On the whole, data gathered thus far suggest that HERG channels may play a prominent role in the control of tumor cell proliferation (7–9) and apoptosis (10). On the other hand, direct proof for the role of HERG channels in the regulation of tumor progression is lacking thus far, except for the demonstration of a functional association of HERG channels with integrin receptors (11, 12), which are the adhesion receptors mainly involved in tumor cell migration and invasion (1, 2).

Colorectal cancer is the third leading cause of cancer death worldwide (13), and the genetic steps involving mutations of suppressor genes (14–16) are well known. On the other hand, there is a paucity of molecular predictive indicators for regional disease invasion and metastasis in this type of cancer.

On the basis of these premises, we studied the expression of HERG channels and their role in the process of tumor progression in colorectal cancers.

MATERIALS AND METHODS

Cell Culture. Human colon cancer cell lines (H630, HCT8, DLD1, and HCT116) were cultured in RPMI 1640 containing 10% FCS and antibiotics (penicillin 100 units/ml and streptomycin 100 μg/ml). H630 cell clones were isolated by limiting dilution. HEK 293 cells were stably transfected with the *herg1* gene in the pCDNA3.1(+) vector (Invitrogen; HEK-HERG1 cells) or with the empty vector (HEK-MOCK). Cells were routinely tested for HERG channel expression by RNase protection assay, Western Blot, and patch clamp (see below).

Tissue Collection. Tissue specimens of paired neoplastic and normal mucosa (collected at least at 10 cm distant from the primary tumor), were obtained from surgical operations in patients suffering from colorectal cancers, after informed written consent was obtained. Patients were all treated at the First Division of General Surgery and Transplantation of Careggi Hospital, Firenze. Patients affected by viral hepatitis or who underwent previous combined neoadjuvant radiochemotherapy were excluded from data collection. Clinicopathological characteristics of patients enrolled in the study (evaluated by L. M.) are reported in Table 1. Eleven polyps obtained from both colonoscopies and surgical operations were also examined. Tissue samples were taken in the operating room and immediately frozen or fixed in 4% formaldehyde in PBS in preparation for being embedded in paraffin. Particular care was taken in sampling normal mucosa to avoid the presence of *muscolaris propria,* which has been demonstrated to express the *herg1* gene and HERG1 protein (17).

Cell Invasion Assay. Cell invasion was studied essentially as reported in (18). Two hundred μl of cell suspension were inoculated into the upper well of a Boyden chamber in the presence or in the absence of the specific I_{HERG} inhibitor Way 123,398 (Way; a kind gift from Dr. Walter Spinelli at Wyeth-Ayerst Research, Princeton, NJ) at 40 μM final concentration. After different times of incubation at 37°C, the migrated cells that remained trapped on the lower surface of the filter were fixed, stained with Diff-Quik stain kit (Dade Behering), and counted.

RNase Protection Assay. RNase protection assay was performed essentially as reported in Crociani *et al.* (9), using the *herg* probe, which is capable of recognizing both the *herg1* and the *herg1b* genes.

Reverse Transcription and PCR. Reverse transcription-PCR for the *herg1* and *gapdh* genes was performed as reported previously (7, 8).

Production of the Anti-HERG1 COOH-Terminus Antibody. The cDNA encoding amino acids 1106–1158 of the HERG1 protein was PCR

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Table 1 *Demographic characteristics and histopathological parameters*

	No. of patients	Percentage
Sex		
M	34	57%
F	26	43%
Localization		
Right	20	33%
Transverse	7	12%
Left	12	20%
Rectum	21	35%
Grading		
G1	4	7%
G2	51	85%
not determined	5	8%
Mucin		
Yes	22	37%
No	38	63%
Tumor-Node-Metastasis stage		
1	10	17%
2	20	33%
3	18	30%
4	12	20%
Metastases		
Yes	8	13%
No	52	87%
Dukes'		
A	10	17%
B	20	33%
C	22	37%
D	8	13%
Jass		
I	2	3%
II	10	17%
III	21	36%
IV	26	44%

amplified (5′ primer: *GTCGAC*GGACTCGCTTTCTCAGGT, *Sal*I site italicized; 3′ primer: *GCGGCCGC*ACTGCCCGGGT, *Not*I site italicized) and cloned in frame with *GST* gene into pGEX 4T-2 vector (Pharmacia). Rabbit immunization, collection, and testing of the serum were performed as reported for the anti-HERG NH_2 terminus antibody (8).

Protein Extraction and Western Blot. Membrane protein extraction from HEK 293, H630, and HCT8 cells was performed as described previously (9). Tissues from both cancerous tissues and the corresponding mucosa were lysed by homogenizing samples in cold Tris HCl (pH 7.4) 50 mM, NaCl 125 mM, NP40 0.1%, EDTA 5 mM, and NaF 0.1 M, plus a complete protease inhibitors mixture (Roche). Western blots were obtained as reported (9), loading different amounts of protein extracts (15 μg for HEK-HERG1 cells, 60 μg for H630, HCT116, and HCT8 cells; and 60 μg for neoplastic and normal tissues), and membranes were decorated with the anti-HERG1 COOH-terminus antibody (see above) at 1:1000 dilution. In some experiments the antibody was preincubated with an excess of the antigenic peptide used for immunization.

Immunohistochemistry (IHC). IHC was performed on 7-μm sections mounted on polylysine-coated slides. After dewaxing and blocking endogenous peroxidases, sections were treated with proteinase K (Roche; 5 μg/ml in PBS) and UltraVBlock solution (LabVision) containing 0.1% Triton X100, and then incubated with the primary antibody (anti-HERG1 COOH-terminus antibody; see above) diluted 1:100 in PBS-UltraVBlock (10:1 v/v) overnight at 4°C. Immunostaining was carried out using a commercially available kit (PicTure Plus kit; Zymed). For the negative control preimmune serum replacing the primary antibody was used.

Patch Clamp Recordings. Patch-clamp recordings were performed on colon carcinoma cell lines at room temperature with an Axopatch 1-D amplifier (Axon Instruments, Foster City, CA), as reported previously (12). The antiarrhythmic drug Way was used at a 1 μM concentration. For data acquisition and analysis, the pClamp hardware and software (Axon Instruments) and Origin (Microcal Software, Northampton, MA) were routinely used.

RESULTS AND DISCUSSION

The *herg1* Gene and HERG1 Protein Are Highly Expressed in Colon Carcinoma Cell Lines. *herg1* gene expression was tested by RNase protection assay in various human colon cancer cell lines (DLD1, HCT8, HCT116, and H630) as performed previously in various cancer cell lines (9). As shown in Fig. 1*A*, the *herg1* gene was expressed in all of the cell lines examined although at different degrees (see Fig. 1, *inset*). In particular, HCT116 and H630 cells expressed *herg1* at the highest level, whereas HCT8 cells expressed it at the lowest level. No expression of the alternative transcript *herg1b* could be detected in colon cancer cell lines, whereas it was present in the heart, as expected (9, 19).

The above results were also confirmed by Western blots with a novel anti-HERG1 COOH-terminus antibody developed in our laboratory. The immunoreactivity and specificity of the antibody was first tested on proteins extracted from *herg1*-transfected HEK 293 cells (Fig. 1*B*). The protein pattern was the same as that obtained with commercial anti-COOH-terminus antibodies (9). When tested on colon cancer cells (Fig. 1*C*), the antibody recognized the HERG1 protein in its immature (M_r 135,000) and mature (M_r ~155,000) forms (9). No HERG1B protein was detectable. These experiments also confirmed that H630 and HCT116 cells express the HERG1 protein at higher levels compared with HCT8 cells.

A typical I_{HERG} could also be recorded in colon cancer cells: a representative example is shown in Fig. 1*D*. The inward current that was observed in high potassium extracellular solution (Fig. 1*D*′), was completely blocked by Way, specific inhibitor of I_{HERG} (Fig. 1*D*″). The current resulting from the subtraction of traces obtained in the presence and absence of Way (Fig. 1*D*‴) displays the typical features of the I_{HERG} described previously in many tumor cell lines (4, 5).

The Activity of HERG Channels Regulates Cell Invasion in Colon Cancer Cells. The role of HERG channels in the invasiveness of colon cancer cells was first studied by analyzing the effect of HERG channel activity on cell migration through Matrigel-coated porous membranes inserted into Boyden chambers. Experiments were performed in the absence or in the presence of the HERG current inhibitor Way (5, 9). As shown in Fig. 2 the addition of Way significantly reduced cell migration in H630, HCT116, and, although to a lesser extent, HCT8 cells; on the other hand, migration of HEK 293 cells, an epithelial cell line devoid of HERG channels, was unaffected by Way addition (Ref. 20; see also Fig. 1*B*).

These data show that HERG activity is an important factor regulating cell invasion of colon cancer cells. Moreover, data on Fig. 2 show a trend of correlation between the amount of HERG1 expressed and the invasion potential in the various cell lines, suggesting that the amount of HERG1 protein could somehow modulate the invasive capacity of colon cancer cells.

The Amount of HERG1 Protein Correlates with the Invasion Capacity of Colon Cancer Cells. Because the cells in Fig. 2 were derived from potentially divergent sources (and may have many genetic differences in addition to their HERG expression), to test whether HERG1 expression could be specifically responsible for the difference in invasive capacity in colonic cancer cells, we developed a set of cell lines with various levels of HERG expression from a homogeneous background. Different clones from the H630 cell line were selected to obtain cells displaying low and high HERG expression. Cloning by limiting dilution yielded ~20 clones, with roughly the same amount of clones with low and high HERG expression. Two clones were chosen displaying both the *herg1* mRNA (Fig. 3*A*) and the HERG1 protein (Fig. 3*B*) at high (clone 3C8) and low (clone 3D3) levels, respectively. A correlation between the amount of HERG1 protein and the average amount of I_{HERG} expressed in the cell population was confirmed (data not shown). When analyzed regarding their migration through Matrigel, the 3C8 clone displayed an invasion capacity almost three times higher than the 3D3 clone (Fig. 3*C*, *black bars*). The invasive phenotype of the 3C8 clone reverted to the less invasive phenotype (associated with low HERG activity) when the

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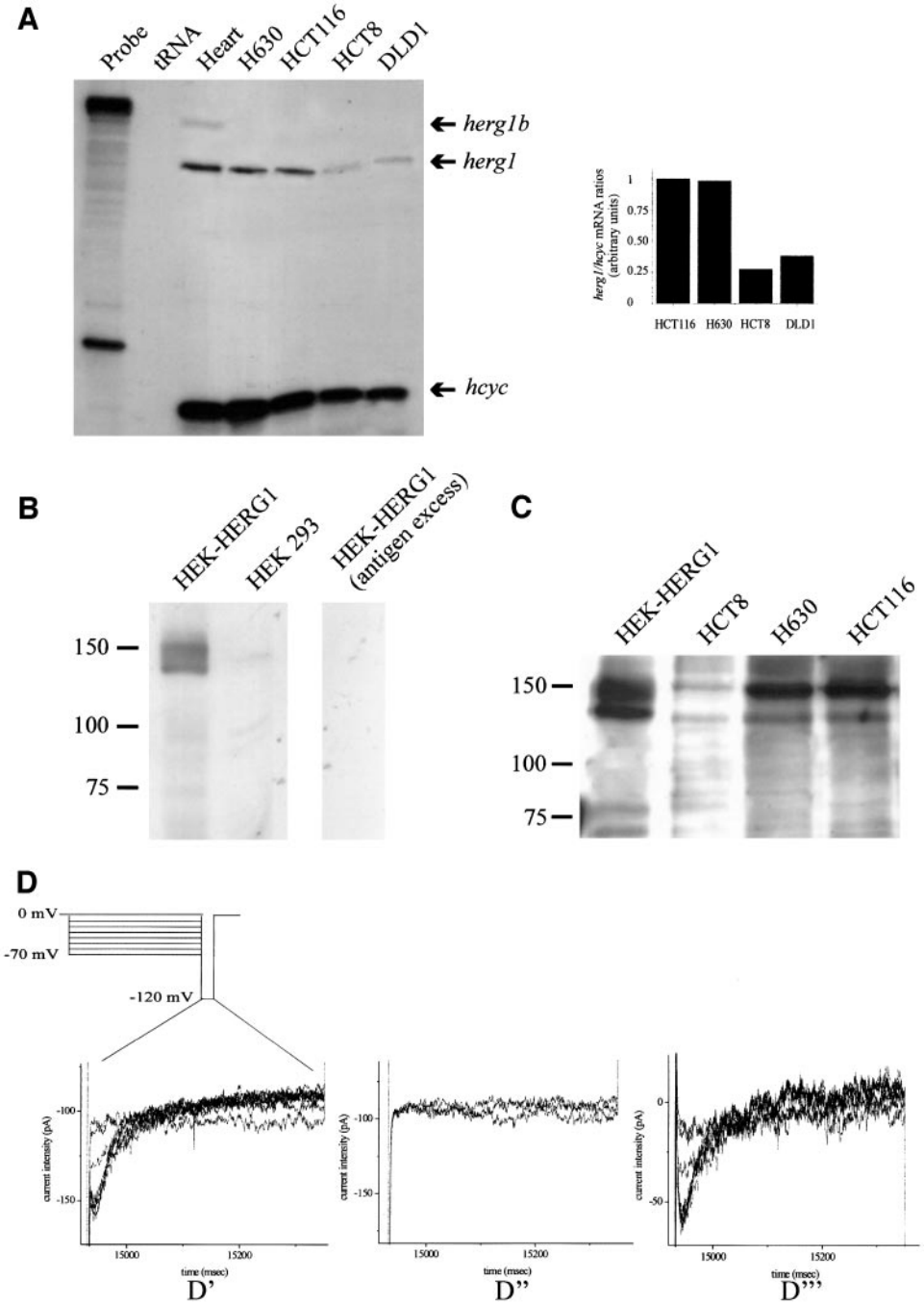

Fig. 1. *herg1* gene and HERG1 protein expression in human colon carcinoma cell lines. *A, herg1* gene expression. RNase protection assay was performed on RNA extracted from DLD1, HCT8, HCT116, and H630 colon carcinoma cells, and probed with the *herg1* probe, which is able to discriminate between *herg1* and *herg1b* as described in (9). Human heart RNA was used as a control; human *cyclophilin* (*hcyc*; Ambion) was used as an internal control and yeast tRNA as a negative control to test for probe self protection bands. Exposure was for 1 day. *herg1, herg1b,* and *hcyc* protected bands are indicated by *arrows*. *Inset,* densitometric analysis of *herg1* expression; signals of the *herg1* protected bands were normalized using the corresponding values of *cyclophilin,* and the value obtained in HCT116 cells was normalized to 1. *B,* HERG1 protein expression in HEK 293 cells; Western Blots were performed on proteins extracted from HEK 293 cells transfected with a *herg1* construct (HEK-HERG1; *Lane 1*) and from HEK 293 wild-type cells (*Lane 2*), and were then probed with the anti COOH-terminus antibody as described in "Materials and Methods." *Lane 3* shows the result of a Western blot performed on HEK-HERG1 using the antibody preincubated with an excess of antigenic protein. *C,* HERG1 protein expression in colon carcinoma cells lines. A Western blot was performed as in *B. Lane 1*: HEK-HERG1; *Lane 2*: H630; *Lane 3*: HCT8; *Lane 4*: HCT116. *D,* HERG current traces obtained in H630 colon carcinoma cell line: I_{HERG} was measured at $[K^+]_o = 40$ mM, as the peak current elicited at -120 mV, after 15 s preconditioning at 0 mV. *Top,* protocol used in the experiment shown. (*D′*) traces obtained in control conditions; (*D″*) traces obtained after perfusion of the cell examined with 1 μM Way; (*D‴*) traces obtained after subctracting traces of *D″* from traces of *D′* and revealing the net I_{HERG}. Note the different *X*-axis scale in *D‴*.

activity of the HERG channels was specifically blocked by Way addition (Fig. 3*C, white bars*). The difference in both HERG expression and invasion capacity of the two clones was maintained between 4 and 20 passages, and after freezing and thawing. On the other hand no difference in the growth in soft agar of the two clones was ever detected (10.8 ± 2.9 large colonies/microscopic field in 3D3 *versus* 11.0 ± 7.0 in 3C8).

The relationship between HERG1 expression and invasion capacity was confirmed by experiments on HEK 293 cells transfected with either a *herg1*-containing plasmid (HEK-HERG1) or an empty vector (HEK-MOCK). As shown in Fig. 4, cell migration through Matrigel was strongly increased in HEK 293 cells overexpressing *herg1* as compared with HEK-MOCK. Here again cell invasion turned out to depend on HERG channel activity (see the inhibitory effect of Way on HEK-HERG1 cells reported in Fig. 4, *inset*). It is worth noting that Way addition did not alter cell invasion capacity in HEK-MOCK cells (data not shown), as stated above for HEK 293 wild-type cells.

***herg1* Gene and HERG1 Protein Are Expressed in Primary Human Colorectal Cancers, with a Higher Incidence in Metastatic Cancers.** To assess the pathophysiological significance *in vivo* of the results gathered thus far, we investigated whether HERG channels were expressed also in primary colorectal cancers, and if a correlation existed between such expression and the acquisition of an invasive and metastatic phenotype.

The expression of the *herg1* gene and protein was determined in a set of primary colorectal adenocarcinomas (Adk) displaying different anatomical localizations and tumor grades (see Table 1). For each tumor, a sample of the corresponding normal colonic mucosa (M) was

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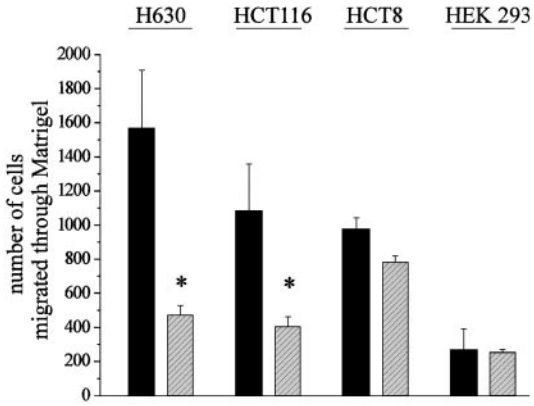

Fig. 2. Role of HERG1 in invasion of colon carcinoma cells, effect of Way. Colon carcinoma cell lines (H630, HCT116, and HCT8) and HEK 293 cells were seeded onto Matrigel-coated porous membranes inserted into Boyden chambers, in RPMI 1640 containing 250 μg/ml BSA, and left to migrate for 6 h (H630, HCT 116, and HCT8) or 16 h (HEK 293) at 37°C in a humidified atmosphere containing 5% CO_2. The specific HERG inhibitor Way was added at time zero to the cell suspension at 40 μM final concentration; this concentration has been used previously to impair leukemia and neuroblastoma cell proliferation (7, 9). Values are reported as the total number of migrated cells, and represent means of three separate experiments performed, each carried out in triplicate; *bars*, ±SE. * $P < 0.005$ Student's *t* test.

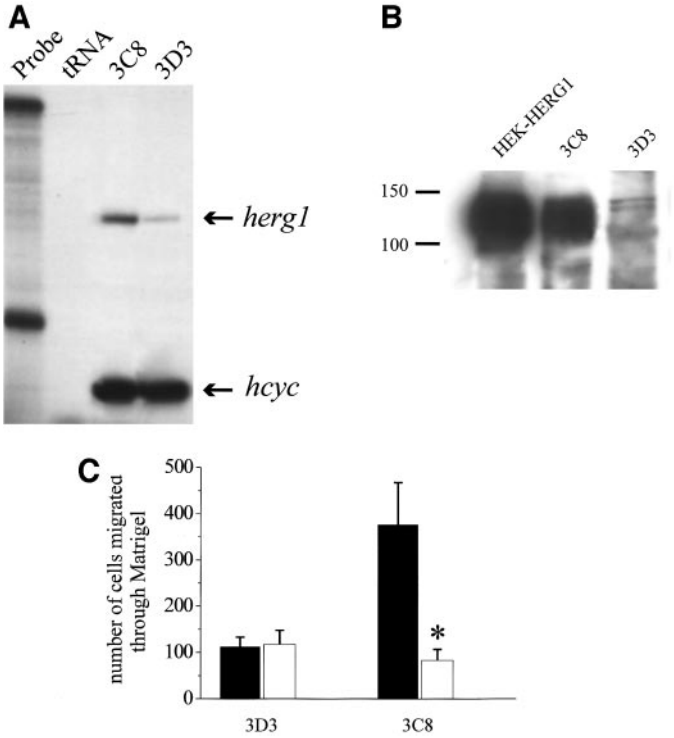

Fig. 3. Relationship between HERG1 expression and invasion capacity in colon carcinoma cells. *A* and *B*, selection of different clones from H630 cells with high and low *herg1* gene (*A*) and HERG1 protein (*B*) expression. Different clones from H630 cells were isolated by limiting dilution, and the amount of *herg1* gene and HERG1 protein expression was analyzed by RNase protection assay and Western blot, as reported in the legend to Fig. 1. Two clones with high (*3C8*) and low (*3D3*) expression of both the gene and the protein are reported. *C*, invasion capacity of H630 cell clones expressing HERG1 at different levels. The invasion potential of 3C8 and 3D3 clones was studied by measuring the number of migrated cells as reported in the legend to Fig. 2, except that cells were left to migrate for 3 h in the absence (■) or in the presence (□) of the specific HERG inhibitor Way. Data represent the mean of four experiments, each carried out in triplicate; *bars*, ±SE. * $P < 0.005$, Student's *t* test.

also included in the study; some adenomas (Ad) obtained either from surgical operations or endoscopies were also studied. All of the samples were analyzed for *herg1* gene expression by reverse transcription-PCR, and HERG1 expression by Western blot and IHC.

As shown in Fig. 5*A* the PCR band related to the *herg1* transcript was easily detectable in representative samples belonging to Adk, whereas a corresponding band was undetectable both in M and Ad. This result was confirmed by Western blot experiments: a typical experiment is reported in Fig. 5*B*. The molecular weight of the band was M_r 145,000, as described in human hearts by Pond *et al.* (21). Here again HERG1B was not expressed.

A complete IHC analysis of HERG1 protein expression on paraffin sections of all of the cases collected was performed. As shown in Fig. 6 no specific signal relative to HERG1 protein was evident either in epithelial tubules of M (Fig. 6*A*) or in Ad (Fig. 6*B*); on the other hand, Adk samples were positive for HERG1 (Fig. 6*C*), showing a specific labeling of epithelial cells. A focal pattern of staining was always

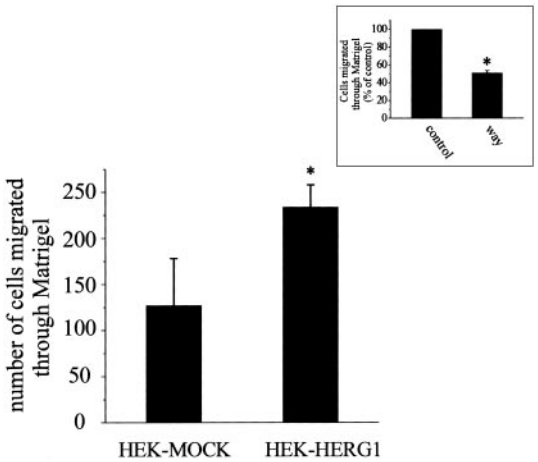

Fig. 4. Effect of HERG1 overexpression on invasion by HEK 293 cells. HEK 293 cells were stably transfected with *herg1*-containing (*HEK-HERG1*) or an empty pCDNA3.1 vector (Mock transfected, see "Materials and Methods"). Cell invasion was studied as reported in the legend to Fig. 2 except that the cells were left to migrate for 12 h. Values are means of four separate experiments each carried out in triplicate; *bars*, ±SE. * $P < 0.005$, Student's *t* test. *Inset*, the effect of Way addition on HEK-HERG1 migration.

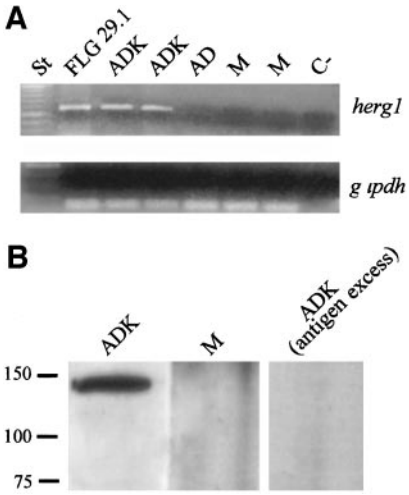

Fig. 5. *herg1* gene and HERG1 protein expression in primary human colorectal cancers. *A*, *herg1* gene expression. Reverse transcription-PCR relative to *herg1* and *gapdh* genes from colorectal cancer cDNA was performed as reported previously (10). The FLG 29.1 cell line was used as the positive control for *herg1* expression; *ADK*, adenocarcinoma sample; *AD*, adenoma sample; *M*, normal colonic mucosa sample of the two ADK reported in the figure; *C*−: no DNA, no RNA sample. *B*, HERG1 protein expression in neoplastic and normal tissue. A Western blot was performed on primary human tissue as reported in "Materials and Methods" using the anti-HERG 1 antibody produced in our laboratory. *ADK*, adenocarcinoma sample; *M*, normal colonic mucosa sample; *ADK* (antigen excess), adenocarcinoma sample probed with the anti-HERG 1 antibody preincubated with an excess of antigenic protein. Note that no band below the HERG1 band (in the expected position of HERG1B) is detectable.

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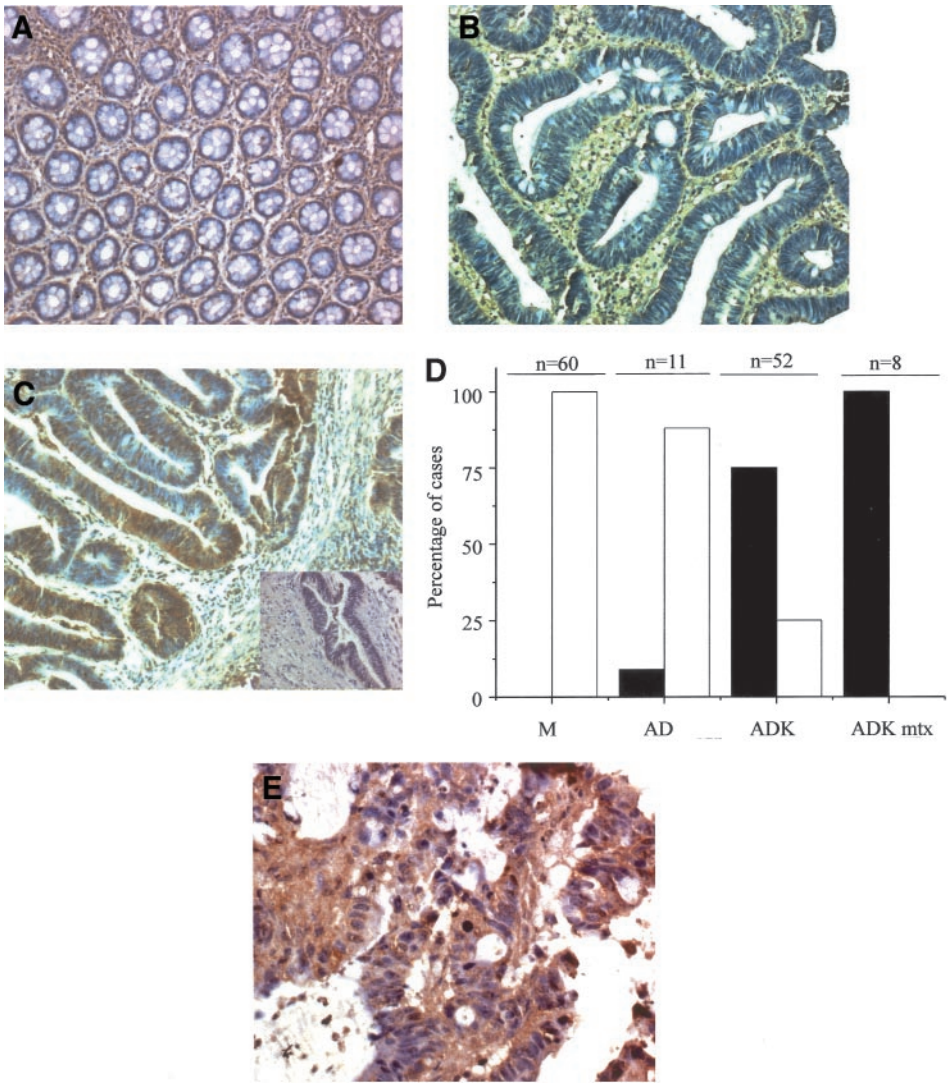

Fig. 6. Immunohistochemical detection of HERG1 protein on normal and neoplastic colorectal tissues. Immunohistochemical detection of HERG1 protein on paraffin-embedded normal and neoplastic human colorectal tissues was performed as reported in "Materials and Methods," using the anti-HERG 1 polyclonal antibody reported above, and used at 1:100 dilution. *A*, normal colonic mucosa, normal epithelial tubules resulted in no HERG-specific staining. *B*, colonic adenoma, with no HERG-staining in tubular structures. *C*, adenocarcinoma of the rectum. Note neoplastic epithelial cells showing an intense and focal immunostaining; in the *inset* is reported a neoplastic specimen decorated with preimmune serum diluted 1:50. *D*, histogram showing data relative to all of the specimens collected (see Table 1). Sixty samples of adenocarcinoma (*ADK*) and paired colonic mucosa (*M*) were examined: 8 ADK were classified as Dukes' D (*ADK Mtx*), whereas the others belonged to Dukes' groups *A–C*. Eleven adenomas (*AD*) were also examined in this study. *E*, adenocarcinoma metastasis to the liver. Almost all of the metastatic epithelial cells show an intense immunostaining, without the focal pattern displayed by primary tumors (see *C*). In this case the anti-HERG1 antibody used was diluted 1:200.

detected in positive Adk samples. Control experiments performed with preimmune serum resulted in no positive staining (a representative example of an Adk sample is shown in Fig. 6*C*, *inset*).

The comparison of data obtained by reverse transcription-PCR and IHC resulted in essentially concordant results: a quantitative analysis of all of the results obtained is reported in Fig. 6*D* and shows that the HERG1 protein was never expressed in M (60 of 60 specimens examined), but was expressed in 9% (1 of 11) Ad, and in 75% (39 of 52 cases) of nonmetastatic Adk (Dukes' A-C). Metastatic (Dukes' D) Adk turned out to be 100% positive for HERG1 protein expression (8 of 8). Hepatic and peritoneal metastases (Fig. 6*E*) not only were positive, but also displayed a widespread and homogeneous staining (rather than showing a focal pattern of HERG1 expression).

CONCLUSIONS

We studied the role of HERG channels in colon cancer cell invasiveness, and showed that: (*a*) almost all of the colon cancer cell lines express the *herg1* gene and HERG1 protein; (*b*) the activity of HERG channels regulates cell invasiveness in many colon cancer cell lines; (*c*) the amount of HERG1 protein correlates with a more invasive phenotype of colon cancer cells; and (*d*) whereas no expression of HERG channels was detected in normal human colonic mucosa and adenomas, a high percentage of primary colorectal cancers expressed the HERG channel, with a higher incidence in metastatic cancers. Therefore, these results provide evidence that K^+ channels belonging to the HERG family are involved in the establishment of an invasive phenotype in colorectal cancer cells both *in vitro* and *in vivo*.

Data reported in this article point to the conclusion that the role of HERG channels in primary solid tumors like colorectal cancers could be that of determining the acquisition of features leading to the establishment of a true invasive and metastatic cancer. Therefore, the immunohistochemical detection of HERG1 protein could represent an independent prognostic factor in the future.

On the whole, voltage-dependent K^+ channels, especially those belonging to the HERG subfamily, could represent oncogenic products, because they promote cell proliferation and survival (7–10), and contribute to conferring a malignant, invasive phenotype to cancer cells.

Targeted therapies promise to revolutionize the care of cancer patients: HERG channels and their inhibitors could be used not only as novel prognostic factors but also as novel molecular targets in the future.

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