Human Cancer Biology

Clinical Cancer Research

hERG1 Channels Regulate VEGF-A Secretion in Human Gastric Cancer: Clinicopathological Correlations and Therapeutical Implications

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Abstract

Purpose: hERG1 channels are aberrantly expressed in several types of human cancers, where they affect different aspects of cancer cell behavior. A thorough analysis of the functional role and clinical significance of hERG1 channels in gastric cancer is still lacking.

Experimental Design: hERG1 expression was tested in a wide (508 samples) Italian cohort of surgically resected patients with gastric cancer, by immunohistochemistry and real-time quantitative PCR. The functional link between hERG1 and the VEGF-A was studied in different gastric cancer cell lines. The effects of hERG1 and VEGF-A inhibition were evaluated *in vivo* in xenograft mouse models.

Results: hERG1 was positive in 69% of the patients and positivity correlated with Lauren's intestinal type, fundus localization of the tumor, G1–G2 grading, I and II tumor—node—metastasis stage, and VEGF-A expression. hERG1 activity modulated VEGF-A secretion, through an AKT-dependent regulation of the transcriptional activity of the hypoxia inducible factor. Treatment of immunodeficient mice xenografted with human gastric cancer cells, with a combination of hERG1 blockers and anti-VEGF-A antibodies, impaired tumor growth more than single-drug treatments.

Conclusion: Our results show that hERG1 (i) is aberrantly expressed in human gastric cancer since its early stages; (ii) drives an intracellular pathway leading to VEGF-A secretion; (iii) can be exploited to identify a gastric cancer patients' group where a combined treatment with antiangiogenic drugs and noncardiotoxic hERG1 inhibitors could be proposed. *Clin Cancer Res; 20(6); 1502–12. ©2014 AACR.*

Introduction

Despite the decrease in gastric cancer mortality observed worldwide in the last decades, gastric cancer is still an important health issue (1). Standard chemotherapy, both in resectable and advanced disease, has limited efficacy, therefore the identification of new molecular markers to improve prognosis as well as of mechanisms and targets for therapeutic interventions, are needed (2).

In the last years, ion channels and transporters have been demonstrated to control many key aspects of neoplastic progression in different types of human cancers (3–5). Moreover, blocking the activity of either ion channels or transporters impairs the growth of some tumors, both *in vitro* and *in vivo*. These observations have opened a new field for pharmaceutical research in oncology (6).

In this context, several research groups provided evidences that a pivotal role in cancer progression is exerted by K^+ channels of the *ether à-go-go gene* (EAG) family (7). In particular, we demonstrated that K^+ channels encoded by the *human ether à-go-go-related gene 1* (hERG1) are over- and mis-expressed in human cancers of different histogenesis. In such cells, hERG1 channels control several aspects of the neoplastic cell physiology (7, 8). More importantly, in this view of the purpose of this article, hERG1 activity is modulated by hypoxia (9) and has an important role in

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

In gastric cancer, standard chemotherapy, both in resectable and advanced disease, has limited efficacy. In search of molecular markers to improve prognosis and identify novel therapeutic interventions, we studied hERG1 channels in a wide cohort of gastric cancer samples collected from different Italian centers. We provide evidence that hERG1 is expressed in the majority of samples, especially in Lauren's intestinal type. hERG1 was expressed since the early stages of gastric cancer progression and could identify patients with high-risk T1 stage. We also show that hERG1 regulates VEGF-A secretion in gastric cancer, and that a combined treatment of mice xenografted with gastric cancer cells with hERG1 blockers and anti–VEGF-A antibodies has an additive antitumoral effect. Thus, there is the potential for a personalized treatment combining noncardiotoxic hERG1 blockers and antiangiogenic drugs in patients with hERG1-positive gastric cancer.

regulating VEGF-A secretion in astrocytomas (10). Moreover, hERG1 modulates VEGF-receptor-1 (FLT-1)-induced cell migration and signaling in acute myeloid leukemias (11).

The expression of hERG1 in gastric cancer has been addressed by different groups. It was first shown that hERG1 channels are functionally expressed in gastric cancer cell lines, where they are critical for *in vitro* cell proliferation (12, 13). More recently, hERG1 expression was found to correlate with tumor grading, TNM stage, and lymph node involvement (14) as well as serosal and venous invasion (15), in 2 small cohort retrospective studies. Despite these results, consistent evidences about hERG1 clinical significance in gastric cancer and its prognostic impact are still lacking.

The purpose of this article is to better analyze the expression of hERG1 channels, as well as its prognostic role in a wide Italian cohort of gastric cancer, with peculiar emphasis to its functional correlation with VEGF-A. Moreover, the possible therapeutic effect of combining hERG1 and VEGF-A–targeting treatments in gastric cancer was also investigated.

Materials and Methods

Patients and specimens

Tissue samples ($n = 190$) were obtained after informed written consent from patients who underwent radical surgery for primary gastric cancer at the Department of Surgery and Translational Medicine, University of Florence and the General Surgery and Surgical Oncology, Azienda Ospedaliero-Universitaria, Careggi. Samples were collected as in ref. 16. All samples were divided into 3 aliquots, 1 immediately fixed in formalin, 1 frozen in liquid nitrogen for storage, and the other stored in RNAlater (Ambion).

Moreover, a multicenter cohort of gastric cancer archival samples ($n = 389$) mainly assembled as tissue microarrays was collected as specified in Supplementary Data. Patients were enrolled between 1987 and 2008 and their lesions encompassed all disease stages. Subjects who had undergone preoperative radiotherapy or chemotherapy were excluded. Considering both the prospective and the retrospective cohorts, 579 samples were analyzed. Diagnosis and histologic grading were assessed using standard criteria by experienced pathologists (L. Messerini, A. Tomezzoli, C. Vindigni, and L. Saragoni).

Immunohistochemistry

hERG1 and VEGF expression were retrospectively tested in 579 patients by immunohistochemistry (IHC), performed as previously reported (17) using the antibodies reported in Supplementary Table S1. Stained sections were analyzed as in ref. (17).

Statistical analysis

To avoid the exclusion of cases with missing data, the multiple imputation method was used (10 imputations; see Supplementary Data for further details). Statistical analyses were performed by L. Boni using SAS version 9.2 (SAS Institute).

DNA methylation studies

The DNA methylation status of the CpG islands located within the *hERG1A* promoter (18) and next to its transcription starting site (TSS) was determined by chemical modification of unmethylated cytosine to uracil and subsequent PCR using primers specific for either methylated or unmethylated DNA. For details see Supplementary Data. To amplify the promoter and the TSS regions of the *hERG1A* gene on the sodium bisulfite–treated DNA sample, specific primer (designed with the MethPrimer software) were used (Supplementary Table S2).

RNA extraction and reverse transcription

Total RNA was extracted using TRizol (Invitrogen), following the manufacturer's protocol. Reverse transcription (RT) was performed using 1 μg of total RNA and Superscript II (Invitrogen), according to the manufacturer's instructions but avoiding the use of reducing agents (dithiotreithol).

Real-time quantitative PCR

RNA extraction, reverse transcription, and real-time quantitative PCR (RQ-PCR) were performed as in ref. 11. Further details are reported in Supplementary Data. The primer sequences are reported in Supplementary Table S2. For mRNA stability experiments, data were normalized to 18S rRNA, whose net amount is not affected by actinomycin D (ActD) treatment.

Cell cultures

All the cell lines used and culture conditions are listed in Supplementary Table S3.

VEGF-A secretion

Cells were seeded into 24-well cell culture plates at 2×10^5 cells/mL in standard culture medium. After 24 hours, the medium was removed and 0.5 mL of Optimem (Gibco) was added. After an additional 24 hours incubation, the medium was collected and VEGF-A measured using the DuoSet ELISA Development System (R&D Systems). Cells were recovered and counted to normalize the VEGF-A secretion data. When needed, the following inhibitors reported in parentheses were added along with Optimem: (i) hERG1-specific inhibitors [E4031 or WAY 123,398 (WAY), at the final concentration 40 μmol/L, as described in ref. 10]; (ii) the PI3K/Akt inhibitor LY294002 (10 μmol/L; Sigma), or the PI3K/Akt inhibitor perifosine (20 μmol/L, kindly provided by Dr. A. Martelli, University of Bologna).

Cell transfection

Transient transfections were commonly performed using the Lipofectamine 2000 reagent (Invitrogen) for siRNAs. For the transfection of Akt1 and Akt2, the Hiperfect Transfection Reagent (Qiagen) was used following the manufacturer's instructions.

Hypoxia-inducible factor activity

Hypoxia-inducible factor (HIF) activity was measured using cells transfected with the hypoxia responsive element luciferase reporter gene vector, kindly provided by Dr. A. Giaccia (Stanford University School of Medicine, Stanford, CA), and measuring luciferase activity. For detailed description, see Supplementary Data.

In vivo experiments on nu/nu mice

All *in vivo* experiments are extensively described in Supplementary Data. All experimentation on live vertebrates described in this article was approved by the Italian Ministry of Health (document no. 140/2009-B).

Results

Analysis of hERG1 expression in primary gastric cancer

To define the clinical significance of hERG1 in gastric cancer, we first carefully evaluated its expression and function in both gastric cancer primary samples and gastric cancer cell lines.

hERG1 protein expression was determined through IHC in gastric cancer primary samples, analyzing both the tumor tissue and the adjacent normal gastric mucosa (Fig. 1). No hERG1 immunostaining was detected in the lining epithelium of the normal mucosa (Fig. 1A). In some samples, in which fundic glands were present, we detected hERG1 positivity in parietal cells (Fig. 1B, see arrows). A strong and diffuse hERG1 immunoreactivity was detected in tumor samples, with a specific expression in neoplastic epithelial cells. This was more evident in Lauren's intestinal type (Fig. 1C), whereas diffuse type gastric cancer were negative to hERG1 staining (Fig. 1D). Figure 1E–H show the hERG1 staining in gastric cancer cases of different grading and stage, whereas low-magnification pictures in which hERG1 focal expression can be better observed are in Supplementary Fig. S1. These data are discussed in the paragraph "Clinical significance of hERG1 in gastric cancer." Western blot experiments performed in some of the samples collected confirmed IHC data (Supplementary Fig. S2). To strengthen these results, hERG1 expression was evaluated in the whole set of samples by IHC using both an anti C-terminus (intracellular) polyclonal antibody (16) and a monoclonal antibody recognizing an extracellular epitope (17). As better analyzed below (see paragraph "Clinical significance of hERG1 in gastric cancer") more than 60% of the samples displayed a high hERG1 immunoreactivity.

We also performed RQ-PCR experiments in order to evaluate whether the altered hERG1 expression in tumor samples correlated with an altered *hERG1* mRNA level. RQ-PCR also allowed us to discriminate between the 2 *hERG1* transcripts, *hERG1A* and *hERG1B* (19). Figure 2A shows RQ-PCR data relative to the *hERG1A* transcript, obtained in a subset of the collected specimens ($n = 28$). Data are expressed as folds of expression, compared with the corresponding normal mucosa. The *hERG1A* transcript showed a variable expression and was expressed at high levels in roughly 50% of gastric cancer samples. However, the *hERG1B* transcript was never expressed at levels comparable or higher than the normal mucosa (Supplementary Fig. S3).

To gain insights on the genetic mechanisms underlying *hERG1A* overexpression in gastric cancer, we performed molecular analyses using different gastric cancer cell lines as a model. As shown in Fig. 2B, the *hERG1A* transcript was expressed in all the gastric cancer cell lines, although at variable levels, from more than 100 folds (AKG cells) to nihil (AGS cells; Fig. 2B). No expression of the *hERG1B* isoform was detected in any of the gastric cancer cell lines tested (not shown; ref. 12). These results were confirmed by Western Blots performed on membrane extracts (see Supplementary Fig. S4). Moreover, a typical I_{hERG} was recorded in those cell lines with a significant hERG1 expression. A representative example, relative to KATO III cells, is reported in Fig. 2C. As a whole, 2 of the 4 examined gastric cancer cell lines showed high hERG1 expression, with a percentage mimicking results obtained in gastric cancer primary samples.

We also analyzed pre- and posttranslational mechanisms that could underlie the different *hERG1A* expression in gastric cancer cells and primary samples. The relevance of posttranslational mechanisms was excluded, because no differences in the amount of the $hERG1_{USO}$ protein (ref. 20; e.g., the main posttranslational mechanism affecting hERG1 protein levels) were detected (Supplementary Fig. S5). We then analyzed the methylation status in a subset of gastric cancer primary samples ($n = 13$). To this purpose, 7 samples expressing (see asterisks in Fig. 2D) and 6 nonexpressing the *hERG1A* transcript were analyzed, looking at 2 CpG islands, 1 located within the promoter and 1 adjacent to the TSS. As shown in Fig. 2D, primary samples showed a variable methylation status of the CpG island inside the *hERG1A* promoter that was independent from the

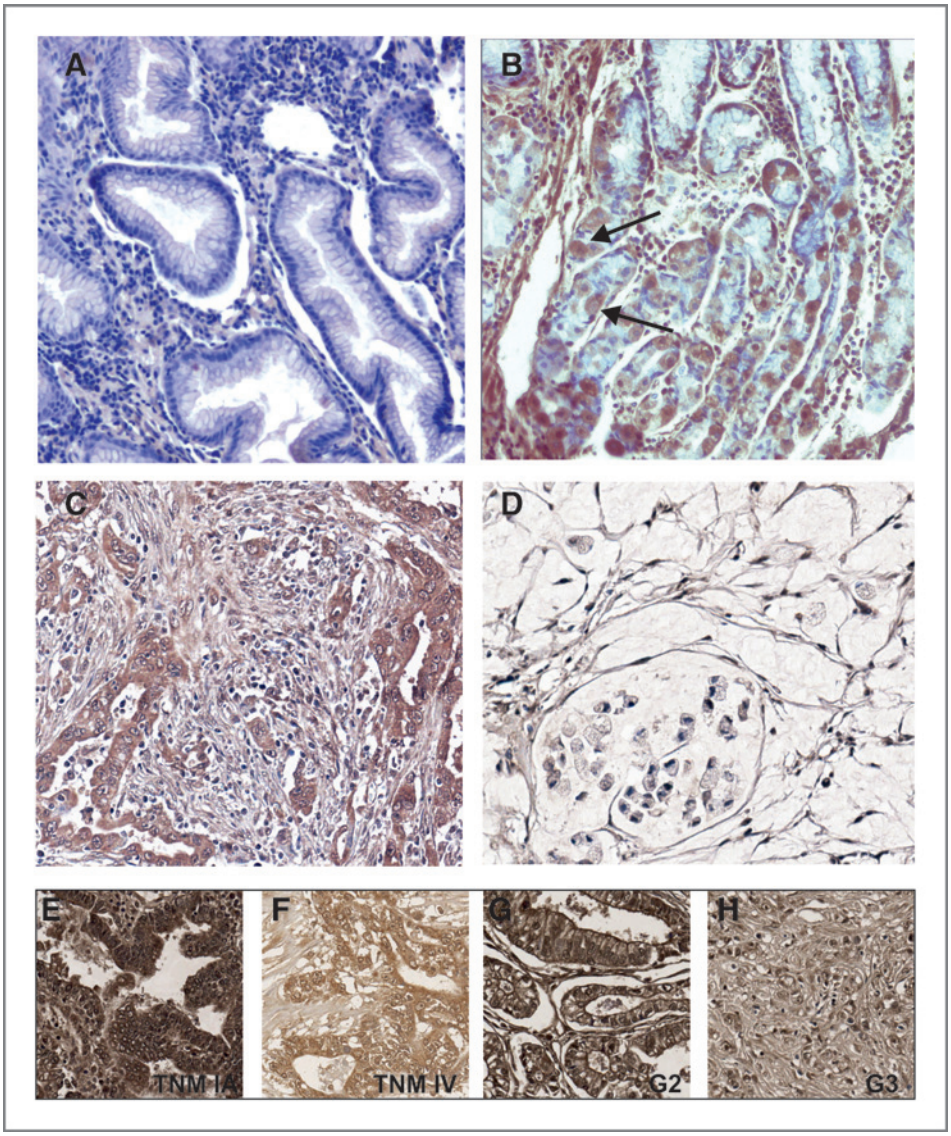

Figure 1. hERG1 protein expression in primary gastric cancer samples. IHC was performed on gastric cancer samples and paired healthy mucosa. A and B, staining of normal lining mucosa and fundic cells. Some of gastric gland cells (i.e., parietal cells) show a strong expression of hERG1 protein (see arrows) in striking contrast to the lining epithelium. C, microphotograph of a representative sample of the intestinal type showing a strong hERG1 expression in the cytoplasm and on plasma membrane. D, representative IHC performed on a sample of Lauren's diffuse type, negative for hERG1 expression. E–H, hERG1 expression in gastric cancer samples of different TNM stage and grading. Four representative microphotographs are reported, showing hERG1 expression (evaluated with polyclonal antibody) in samples classified as TNM IA and IV, G2, and G3 as indicated in the pictures. Magnification, ×20.

expression of the *hERG1A* gene. However, the CpG island located at the *hERG1A* TSS turned out to be homogeneously unmethylated, a fact that suggests a constitutively active promoter in all the samples tested. As a whole, the methylation levels of the 2 CpG islands analyzed does not seem to explain the different *hERG1A* levels in gastric cancer primary samples.

We then studied *hERG1A* mRNA stability, quantifying *hERG1A* mRNA by RQ-PCR after actinomycin D (ActD) addition. These experiments were performed on the 2 cell lines expressing hERG1 at the highest (AKG) and at the lowest (AGS) levels. After exposure to ActD for either 2 or 6 hours, a greater amount of *hERG1A* mRNA is detectable in AKG compared with AGS cells (Fig. 2E). Hence, an increased mRNA stability (witnessed by a slower rate of mRNA decay) could underlie the *hERG1A* overexpression in gastric cancer cell lines. This finding could be translated to gastric cancer primary samples.

hERG1 channels drive VEGF-A secretion in gastric cancer

We then evaluated the functional role of hERG1 channels in gastric cancer cells. In particular, we analyzed whether a functional link between hERG1 and VEGF-A existed in gastric cancer. All the gastric cancer cell lines under study secreted VEGF-A in the culture medium, as determined by ELISA test, but only those with a significant *hERG1* expression (AKG and KATO III) secreted high levels of the protein (see histograms in Fig. 3A).

VEGF-A secretion turned out to be modulated by hERG1, as shown by data obtained either inhibiting hERG1 activity (through specific blockers) or reducing its expression (through siRNAs). Note that hERG1 blockers had no overlapping effects on *hERG1* expression (Supplementary Table S4). Indeed, the addition of either WAY or E4031 significantly decreased VEGF-A secretion in AKG and KATO III cells (Fig. 3B), whereas had no effect in MKN28 and AGS

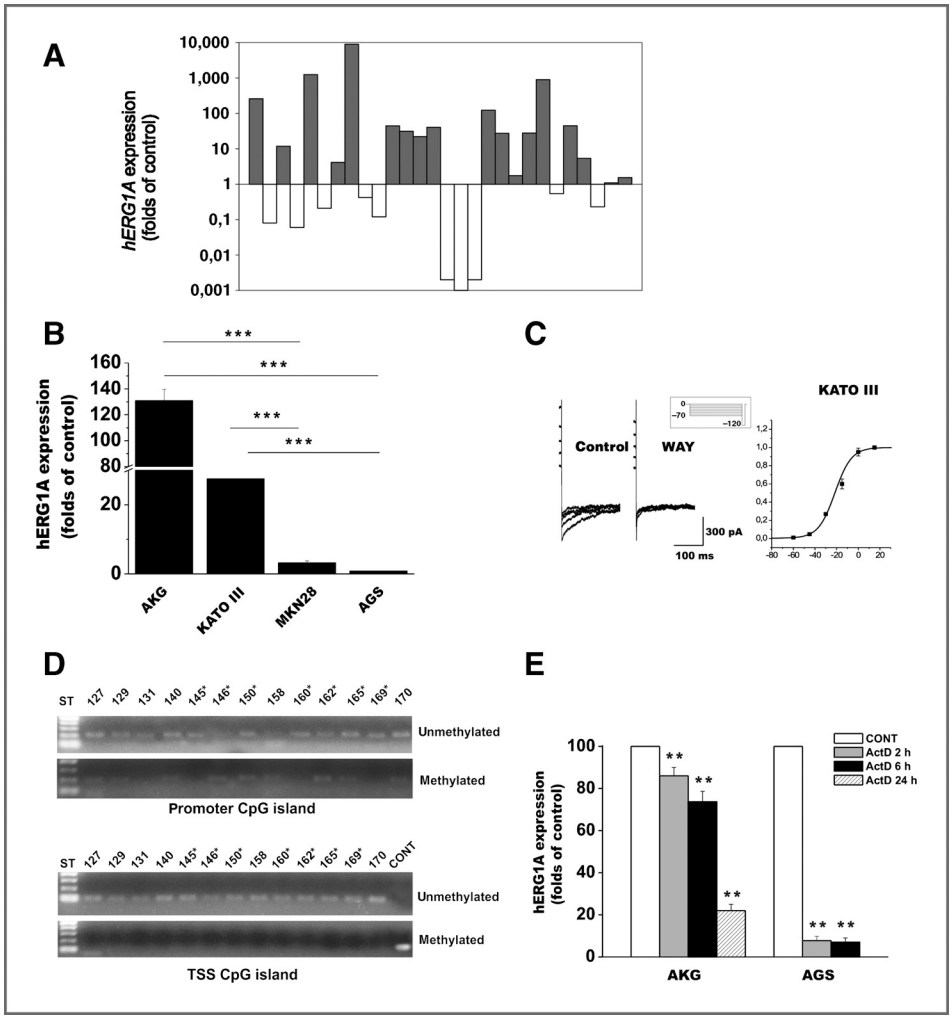

Figure 2. *hERG1A* characterization in primary gastric cancer and cell lines. A, *hERG1A* transcript expression in gastric cancer primary samples. Graph, data obtained by RQ-PCR analysis performed in 28 primary gastric cancer samples and paired healthy mucosa. The detailed procedure is reported in Supplementary Data. Data are normalized on a pool of healthy mucosal samples and *hERG1A* expression is reported as folds of control. B, expression of *hERG1A* in gastric cancer cell lines. In the histogram, data obtained from all the experiments performed on different gastric cancer cell lines are summarized. C, electrophysiological traces registered in KATO III cells. The biophysical profile shows the presence of the hERG1 current. D, RT-PCR results relative to the methylation status of *hERG1A* promoter (top) and TSS CpG island (bottom). Experiments were performed as detailed in Supplementary Data. Asterisks indicate hERG1-expressing samples. E, RQ-PCR experiments performed on AKG and AGS samples treated or not with Actinomycin D (5 μg/mL, after overnight starvation) to inhibit mRNA transcription. Data, means ± SEM of 3 separate experiments, each carried out in duplicate. **, $P < 0.02$; ***, $P < 0.01$ (Student *t* test).

cells. However, tetraethylammonium (TEA), a wide inhibitor of K^+ channels (proven not to affect hERG1 at the concentration used in these experiments), had no effect on VEGF-A secretion (Fig. 3B). To decrease hERG1 expression, 3 different anti-*hERG1* siRNAs (α-siRNAs 1–3) were tested, all effective in reducing *hERG1* expression (Supplementary Table S5). All the α-siRNAs significantly decreased VEGF-A secretion in AKG and KATO III (Fig. 3B). The inhibitory effect of α-siRNAs was identical to that obtained with an anti(α)-*VEGF-A* siRNA (see the last right column relative to AKG and KATO III cells in Fig. 3B).

The decrease of VEGF-A secretion produced by hERG1 inhibition depended on a negative regulation of *VEGF-A* transcription. In fact, α-*hERG1* siRNAs tested either separately (on AKG cells; Supplementary Table S4), or mixed (in both AKG and KATO III cell lines; Fig. 3C), decreased *VEGF-A* expression. The effects of α-*hERG1* siRNAs were not because of off-target effects, because the expression of a completely unrelated transcript, *Kv1.3* (which encodes for a voltage-dependent K^+ channel, often expressed in cancer cells) was totally unaffected by α-*hERG1* siRNAs (Supplementary Table S5). Moreover, the inhibition of *VEGF-A* expression produced by silencing hERG1 channels was similar to that obtained by either blocking hERG1 activity with WAY or silencing *VEGF-A* through α-*VEGF-A* siRNA (Supplementary Table S5).

VEGF-A expression is mainly controlled by the activity of the transcription factor HIF, whose "α" subunit is under control of either O_2 tension or intracellular signaling pathways (21). We recently reported that *VEGF-A* transcription in colorectal cancer cells was controlled by a peculiar signaling pathway triggered by the hERG1/β1 integrin complex, centered on Akt and converging on the regulation of the 2 HIF-α transcripts: *HIF-1α* and *HIF-2α* (22). Hence, we tested whether the same pathway was controlled by hERG1 in gastric cancer cells. We first determined the transcriptional activity of HIF in gastric cancer cells. HIF activity (measured as luciferase activity, see Supplementary Data) was decreased by either E4031 or WAY (Fig. 3D). However, it increased after switching the cells to hypoxia, as expected. HIF activity was also measured quantifying the expression levels of HIF-1α–dependent and HIF-2α–dependent genes. hERG1 inhibition decreased the expression of HIF-1α and HIF-2α coregulated (*GLUT-1*), as well as of HIF-2α

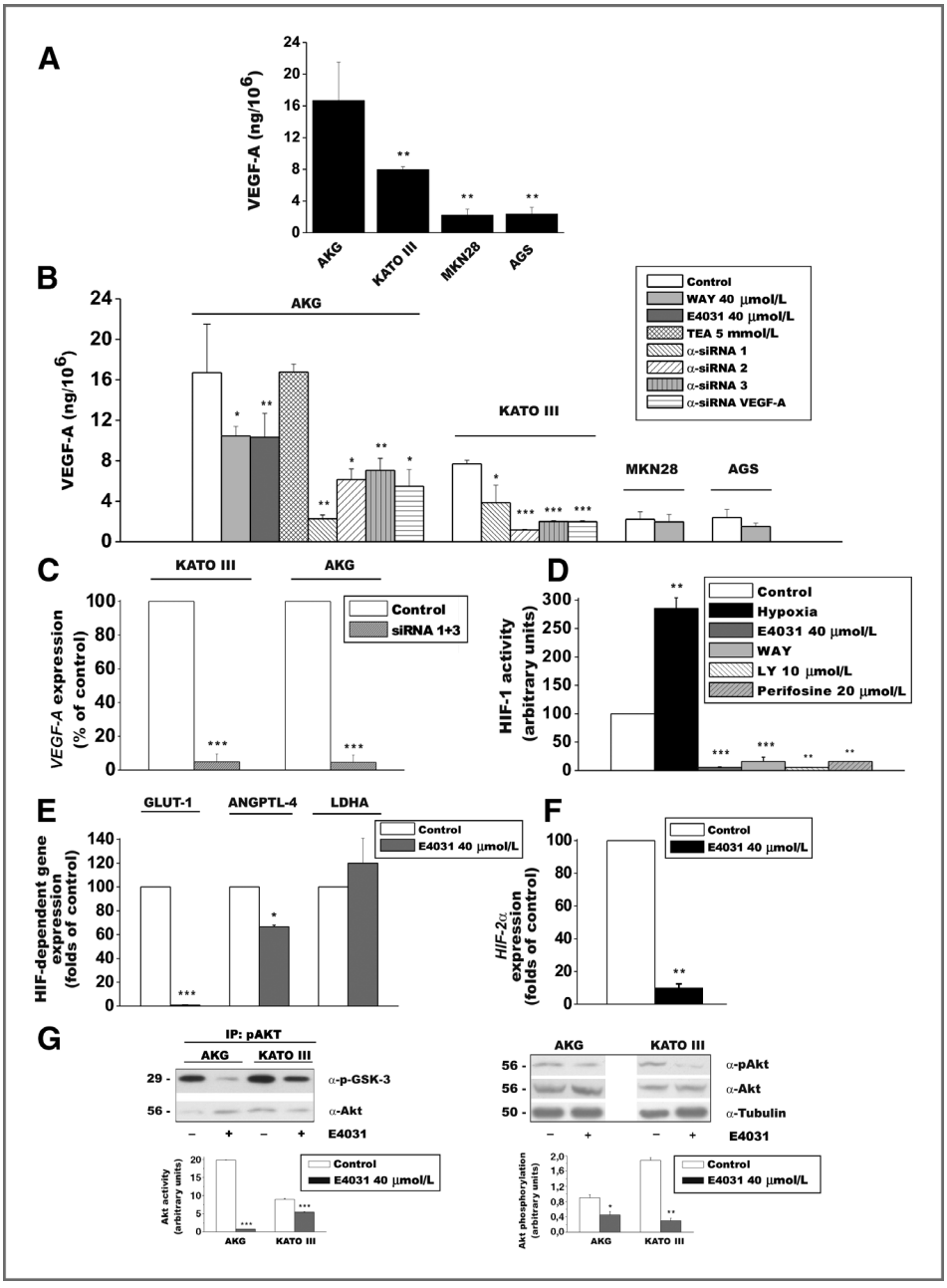

Figure 3. Characterization of hERG1 expression and VEGF-A expression and secretion in gastric cancer cell lines: the effects of hERG1 inhibition or overexpression on VEGF-A secretion. A, VEGF-A secretion in gastric cancer cell lines. In the histogram, data obtained from all the experiments performed on gastric cancer cell lines are summarized. B, the effect of hERG1 blocking on VEGF-A secretion in gastric cancer cells. Ion channel blockers were added 24 hours before VEGF-A measurement. hERG1 inhibitors were used as in ref. 10. Data from 4 different experiments, each carried out in duplicate, are reported as mean ± SEM. TEA data refer to 2 experiments, each carried out in triplicate. C, *VEGF-A* expression in control and hERG1-silenced KATO III and AKG cells. D, normoxic HIF-1α transcriptional activity in AKG cell lines in control conditions and after hERG1A or PI3K/Akt pharmacologic blocking. Hypoxic HIF-1α transcriptional activity was also shown as control. E, fold induction of HIF(s) target genes after hERG1 pharmacologic blocking, *GLUT1*, glucose transporter 1; *ANGPTL-4*, angiopoietin-like 4; *LDHA*, lactate dehydrogenase. F, HIF-2α dependent expression after hERG1 pharmacologic blocking. G, effects of the hERG1 blocker E4031 on Akt activity (left) and on Akt phosphorylation (right) in AKG and KATO III cell lines. Akt activity was evaluated using the Akt Kinase Assay Kit (Cell Signaling) following the manufacturer's instructions. Data, means ± SEM of 2 or 3 separate experiments. *, $P < 0.05$ (Student *t* test). *, $P < 0.05$; **, $P < 0.02$; ***, $P < 0.01$ (Student *t* test).

regulated (*ANGPTL-4*) genes, whereas did not affect the expression of a gene (*LDHA*), whose transcription only depends on HIF-1α (Fig. 3E). Collectively, these data indicate that hERG1 activity modulates mainly HIF-2 transcriptional activity. Consistently, hERG1 blocking significantly reduced the levels of *HIF-2α* transcript (Fig. 3F). HIF activity was also inhibited by 2 different PI3K/Akt inhibitors LY294002 (LY) and perifosine (Fig. 3D), which also significantly decreased VEGF-A secretion (Supplementary Fig. S6). We then measured both Akt activity (by an *in vitro* kinase assay using GSK-3 as a substrate; Fig. 3G, left), and Akt phosphorylation (Fig. 3G, right): both were decreased by hERG1 inhibitors.

On the whole, in gastric cancer cells, hERG1 channels regulate VEGF-A secretion through an Akt-dependent modulation of HIF (mainly HIF-2) transcriptional activity.

Clinical significance of hERG1 in gastric cancer

hERG1 expression was then correlated with clinicopathological parameters as well as with patients' survival in the whole cohort of gastric cancer samples, collected from different Italian centers (see Materials and Methods). From the 579 patients initially considered for the study, 71 were excluded because of incomplete follow up. As shown in Supplementary Table S6, the group of 71 patients excluded from analysis did not significantly differ

from the study population. Patient samples encompassed all TNM stages, with higher percentages in stages III and IV. A slight prevalence of males and G3 pathologic grade characterized the casistic under study (Supplementary Table S6). Moreover, 63.8% of the samples were classified as Lauren intestinal type, which is the most frequent histotype in Italy (23).

All the antibodies were previously validated and negative controls were included in each IHC experiments (a representative picture is reported in Supplementary Fig. S7). For hERG1 expression analyses, data obtained with the hERG1 polyclonal antibody were used (representative pictures are reported in the Supplementary Fig. S8, taking into account 2 scoring groups: lower or higher than 50% (see Materials and Methods in Supplementary Data).

hERG1 was expressed by 69.1% of the samples. hERG1 positivity was more evident in Lauren intestinal type gastric cancer compared with the diffuse type (see also Fig. 1C and D), a finding corroborated by the statistical analysis ($P < 0.0001$; Table 1). Moreover, hERG1 correlated with tumor localization ($P = 0.017$) with a prevalence in the fundus, tumor grading, with a prevalence in G1–G2 ($P < 0.001$; see also panels in Fig. 1) and with the TNM stage ($P = 0.031$). hERG1 positivity was higher in stages I and II (Table 1 and Fig. 1E–H). Finally, a strong correlation with VEGF-A emerged ($P < 0.001$). Often the 2 proteins were coexpressed in the same tissue sample and, more specifically, in the same cancerous epithelial cells, with a similar pattern of expression (see Supplementary Fig. S9).

Table 1. Association between hERG1 expression and clinical and pathologic variables

Variable	hERG1 positivity rate	OR (95% CI)	*P*
All cases	69.1%	—	—
Age, y			
<70	69.3%	1 (ref.)	0.927
≥70	68.9%	0.98 (0.67–1.44)	
Gender			
Male	69.1%	1 (ref.)	0.979
Female	69.0%	1.00 (0.67–1.47)	
Site of primary tumor			
Antrum, cardias	64.8%	1 (ref.)	0.017
Body	68.7%	1.19 (0.75–1.88)	
Fundus	80.4%	2.23 (1.30–3.82)	
Gastric stump, linitis plastica	61.1%	0.85 (0.39–1.84)	
TNM stage			
I	75.3%	1 (ref.)	0.031
II	79.8%	1.30 (0.61–2.75)	
III	65.0%	0.61 (0.33–1.14)	
IV	65.3%	0.62 (0.33–1.17)	
Pathologic grading			
G1, G2	80.9%	1 (ref.)	<0.001
G3, G4	62.3%	0.39 (0.25–0.61)	
Lauren type			
Intestinal	79.0%	1 (ref.)	<0.001
Diffuse	53.4%	0.30 (0.20–0.47)	
Mixed	47.2%	0.24 (0.13–0.43)	
VEGF-A status			
Negative	25.6%	1 (ref.)	<0.001
Positive	75.2%	9.31 (3.61–24.0)	

After a median follow up of 11.1 years (Interquartile Range, IQR = 7.3–15.0), 391 deaths were observed. At the univariate analyses, age >70 years, male sex, site (gastric stump and linitis plastica), advanced stages and diffuse/mixed Lauren were associated with a worse prognosis (Table 2). The multivariate analysis confirmed the results obtained at the univariate analysis (Table 2). No clinically significant interaction emerged between hERG1 expression and the clinical and pathologic parameters (Supplementary Fig. S10). Evaluating the T, N, and M parameters, heterogeneity emerged within T stage ($P < 0.001$, test for interaction). In particular, the interaction analysis showed a statistically significant interaction on overall survival (OS) between T stage and hERG1 expression (HR = 1.51 T1, HR = 0.87 T2, HR = 1.02 T3, HR = 0.64 T4). Hence, we can argue that hERG1 might display a negative prognostic impact in T1 stage patients.

Effects of hERG1 pharmacologic targeting: *in vivo* experiments

Finally, we determined whether hERG1 channels could represent good targets for antineoplastic therapy in gastric cancer. To test this possibility, we analyzed immunodeficient, athymic nu/nu mice subcutaneously injected with hERG1-expressing gastric cancer cells, either AKG or KATO III. In a first set of experiments, mice were injected with AKG cells and treated with the hERG1 inhibitor E4031, daily for 2 weeks starting from the day after inoculum. The masses obtained were then analyzed 5 days after the suspension of treatment E4031 significantly decreased tumor growth, as evidenced by the decrease of the tumor volume (from 277.3 to 19.6 mm^3, $P < 0.05$; Fig. 4A). This effect was paralleled by a significant decrease of tumor angiogenesis, witnessed by intratumoral total vascular area (Fig. 4B). Moreover, vessels within the masses obtained from control, untreated mice were numerous, distinctly small and more homogeneous in calibre (lane "Control" on the right of Fig. 4C), whereas those within the masses from E4031-treated mice were fewer although longer (lane "E4031" on the right of Fig. 4C), with a higher perivascular fibrosis (see the arrow in right). The reduced vasculature of gastric cancer masses of E4031-treated mice was accompanied by a reduction of the expression of VEGF-A and pAkt (Fig. 4C), strongly confirming *in vitro* findings.

Another set of *in vivo* experiments was then performed, injecting KATO III cells and treating the mice when tumor masses reached the volume of 60 mm^3. In these experiments, mice were treated with either E4031 or the anti–VEGF-A antibody (bevacizumab), as single or combined treatments. Tumor growth was inhibited by each of the single treatments as well as by the combination of the 2

Table 2. Univariate and multivariate evaluation of prognostic role for OS of clinical and pathologic variables

	Univariate analysis		Multivariate analysis	
Variable	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
Age, y				
<70	1 (ref.)	<0.001	1 (ref.)	<0.001
≥70	1.63 (1.34–1.99)		2.29 (1.86–2.82)	
Gender				
Male	1 (ref.)	0.012	1 (ref.)	0.004
Female	0.77 (0.62–0.95)		0.73 (0.59–0.91)	
Site of primary tumor				
Antrum, cardias	1 (ref.)	<0.001	1 (ref.)	0.028
Body	1.14 (0.89–1.47)		1.03 (0.80–1.33)	
Fundus	1.37 (1.06–1.76)		1.18 (0.90–1.54)	
Gastric stump, linitis plastica	2.52 (1.67–3.80)		1.96 (1.28–3.00)	
TNM stage				
I	1 (ref.)	<0.001	1 (ref.)	<0.001
II	2.17 (1.40–3.38)		2.33 (1.48–3.66)	
III	4.05 (2.70–6.08)		4.53 (2.97–6.91)	
IV	7.09 (4.68–10.7)		8.42 (5.41–13.1)	
Pathological grading				
G1, G2	1 (ref.)	0.207	1 (ref.)	0.051
G3, G4	1.14 (0.93–1.41)		0.77 (0.59–1.00)	
Lauren type				
Intestinal	1 (ref.)	<0.001	1 (ref.)	0.006
Diffuse	1.55 (1.23–1.94)		1.44 (1.07–1.94)	
Mixed	1.79 (1.30–2.45)		1.74 (1.20–2.51)	
VEGF-A status				
Negative	1 (ref.)	0.510	1 (ref.)	0.575
Positive	1.00 (0.59–1.68)		0.91 (0.62–1.32)	
hERG1 status				
Negative	1 (ref.)	0.726	1 (ref.)	0.119
Positive	0.96 (0.78–1.19)		1.22 (0.95–1.57)	

agents (Fig. 4D, left). After completing the treatment schedule, tumors started to grow again, except in the combined treatment regimen. In particular, when monitored after 10 days of treatment suspension, the mean volume of tumor masses of mice treated with E4031 + bevacizumab was significantly lower than those of mice treated with a single treatment regimen (Fig. 4E). Moreover, strong inhibition of tumor angiogenesis (in this case better witnessed by a decrease of the number of CD34-positive tumor vessels) was observed in masses of mice that underwent the combined treatment (Fig. 4F).

Discussion

This study investigates the functional role and clinical significance of hERG1 potassium channels in gastric cancer. It provides evidence that hERG1 channels are overexpressed at early stages of gastric cancer progression and regulate VEGF-A secretion in gastric cancer. These and other findings support the targeting of hERG1 as a possible patient-tailored antiangiogenic approach in the therapy of gastric cancer.

hERG1 channels turned out to be overexpressed in both primary gastric tumors and gastric cancer cell lines, whereas they were not expressed in the lining epithelium of normal gastric mucosa. In normal stomach samples, we found a high hERG1 IHC positivity in parietal cells of the gastric glands, which indeed express several types of ion channels. In particular, KCNQ1 K^+ channels are expressed on the apical membrane of gastric parietal cells, in conjunction with the accessory beta subunit, KCNE2. The KCNQ1/KCNE2 complex is functional and contribute to acid secretion (24, 25). Although the role of hERG1 channels in gastric parietal cells was out of the scope of our study, it is possible to speculate that they also could be functional in these cells, because KCNE2 behaves also as hERG1 accessory subunit (26).

The hERG1 expression we found in gastric cancer primary samples and cell lines confirms previous data (12, 13). Moreover, we showed that hERG1 is overexpressed and this relies on a higher amount of the *hERG1* transcript (about 20 times more) in neoplastic than in normal gastric mucosa. Particularly, we showed that (i) only the full length *hERG1A*

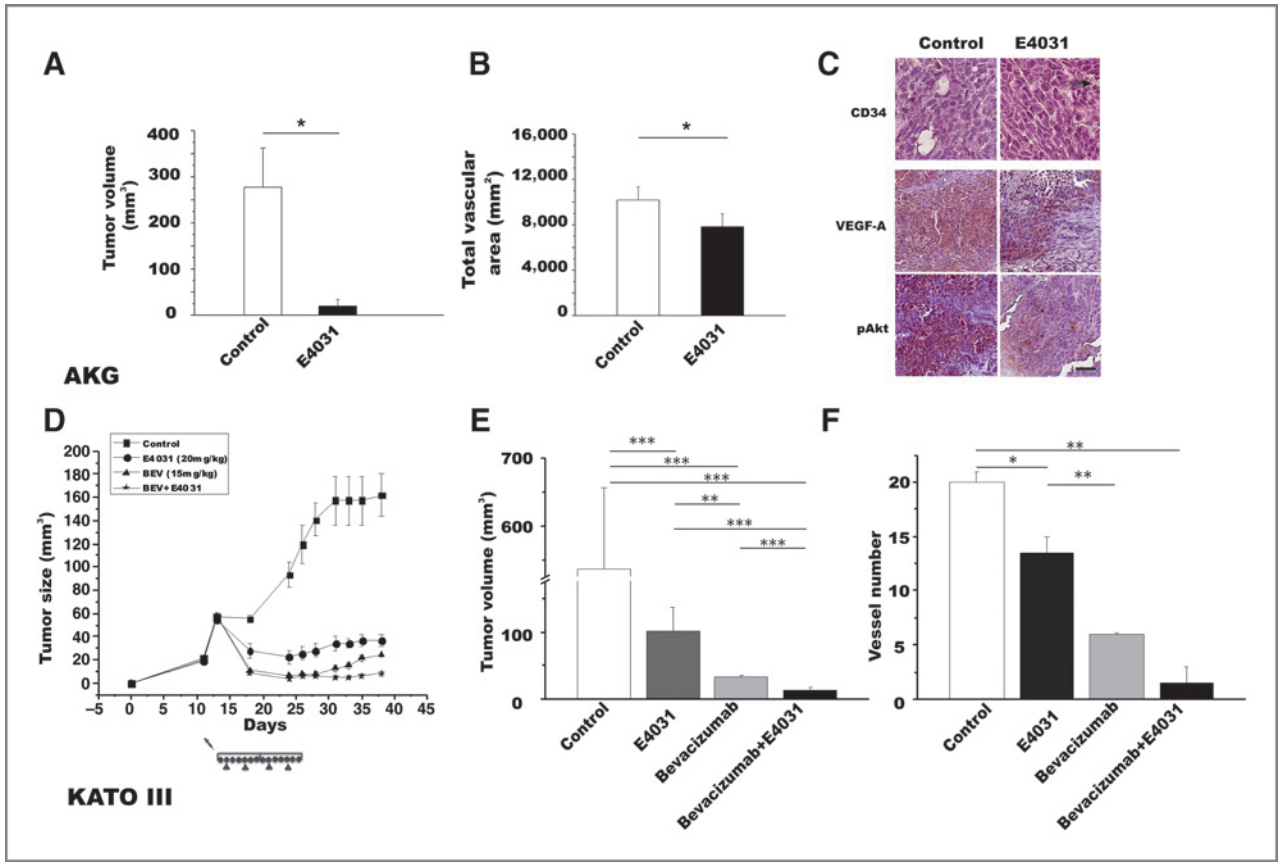

Figure 4. hERG1 channels in gastric cancer as novel therapeutic targets: *in vivo* experiments. A, volume of tumor masses obtained after injection of AKG cells in control (white bar, 277.3 ± 85 mm^3; 246,25 ± 0.095 mm^3) and E4031-treated mice (black bar, 19.6 ± 14.5 mm^3; 103,75 ± 0,05 mm^3). Data, mean of 2 experiments (4 animals/group) ± SEM. B, microvessel density evaluation in tumor masses from Control and E4031-treated mice after injection of AKG cells. Total vascular area was measured as in ref. 11 after staining with an anti-CD34 antibody and is reported as μm^2 per microscopic field. In Control mice, the number of vessels was higher, although not significantly, than in E4031-treated mice (21.6 ± 2.0 vs. 15.1 ± 2.3). As concerning total vascular area, a statistically significant difference emerged between Control and E4031-treated mice (10185.8 ± 1180.8 vs. 7829.4 ± 1148.0). C, histologic analysis of CD34, VEGF-A, and pAkt staining of tumor masses obtained in control and E4031-treated mice after injection of AKG cells. Bar, 200 μm (for CD34) and 100 μm (for VEGF-A and pAkt). For quantification, positively stained cells were counted in 5 randomly selected fields under a magnification of ×400. In Control mice, the percentage of VEGF-A positive cells was higher than in treated animals (45% vs. 20%) and the same occurred for pAkt immunostaining (55% vs. 5%). D–F, mice inoculated with KATO III cells. D, time course of tumor masses growth in the 4 different groups. Treatment schedule is reported below. E, histogram showing tumor volumes of the explanted masses. Control mice, 162 ± 18; E4031-treated animals, 37.25 ± 5; bevacizumab-treated mice, 24.3 ± 0.3; mice treated with bevacizumab + E4031, 8.2 ± 2.3. Data, mean ± SEM. *, $P < 0.05$; **, $P < 0.02$; ***, $P < 0.01$ (Student *t* test). F, histogram showing microvessel number in tumor masses from Control and treated mice after injection of KATO III cells. Control mice, 20 ± 1; E4031-treated animals, 13.5 ± 1.5; bevacizumab-treated mice, 6 ± 0.1; mice treated with bevacizumab + E4031, 1.5 ± 1.5. Data, mean ± SEM. *, $P < 0.05$; **, $P < 0.02$ (Student *t* test).

transcript is overexpressed, a finding completely different from what occurs in other tumors, such as leukemias (11, 19), where only the *hERG1B* transcript is overexpressed. This suggests the existence of a tumor type–related hERG1 isoform signature; (ii) *hERG1A* overexpression in gastric cancer correlates with an increased stability of the corresponding mRNA, in highly hERG1 expressing gastric cancer cells, a fact that candidates this as the mechanism underlying *hERG1A* overexpression in gastric cancer samples. Consistently, we excluded a significant contribution to hERG1 overexpression by the methylation status of the *hERG1A* promoters as well as of post-translational mechanisms, based on the expression of the USO transcripts (20).

The overexpression of hERG1 in gastric cancer is witnessed by a strong immunostaining of gastric cancer samples. In this study, we used 2 different anti-hERG1 antibodies: a polyclonal antibody directed against the intracellular C-terminus of the hERG1 protein and a monoclonal antibody, directed against the S5-P extracellular loop. The 2 sets of experiments gave comparable results, although the concordance was not complete. For mere technical reasons (e.g., the possibility of a lower immunoreactivity of the monoclonal antibody to gastric cells), we favored the use of the polyclonal antibody, whose results well fitted with those obtained measuring *hERG1A* transcript levels by RQ-PCR (Supplementary Fig. S11).

The functional role of hERG1 channels in gastric cancer was analyzed in gastric cancer cell lines and we provided evidence that hERG1 regulates *VEGF-A* transcription and hence VEGF-A secretion in gastric cancer. Hence, hERG1 function in gastric cancer is similar to that discovered in brain tumors (10) and during mouse colorectal carcinogenesis (27). The regulation of VEGF-A secretion occurs exclusively in gastric cancer cells expressing hERG1 at high levels, a fact proven by both pharmacologic and biomolecular hERG1 inhibition. Moreover, such regulation can be traced back to a signaling mechanism triggered by hERG1 and ending into the regulation of HIF transcriptional activity (22). Interestingly, it takes place in normoxic condition when HIF is usually rapidly degraded (21). Moreover, in gastric cancer, the hERG1-dependent pathway mainly impacts onto HIF-2α and the transcription of HIF-2–dependent genes (such as *ANGPTL4*, besides *VEGF-A*), more than of HIF-1–dependent genes, which are mainly related to cell metabolism. We can conclude that, in gastric cancer, hERG1 behaves as a cell-cycle device, capable of regulating cell proliferation (12, 13), as well as a progression-related gene, mainly involved in the regulation of tumor angiogenesis. Although the impact of hERG1 on cell cycle could be traced back to the regulation of intracellular Ca^{2+} levels as a consequence of a hERG1-dependent regulation of the membrane potential value (28), the effects on tumor progression could be related to the hERG1-dependent effect on cell signaling, well documented in several types of cancer (3, 4, 29). This latter ability makes hERG1 not only a canonical ion channel, but also a membrane protein able to influence the expression of tumor-related genes in an unconventional manner. Moreover, the specific impact of hERG1 on HIF-2 regulation in normoxia could put the bases for the development of novel therapeutic strategies.

Finally, we evaluated the clinical significance of hERG1 expression in gastric cancer, studying a large Italian cohort of 508 gastric cancer patients, encompassing different TNM stages. hERG1 expression strongly correlated with intestinal Lauren's histologic type, tumor localization, grading (mainly G1–G2) and TNM stage, with a prevalence in stages I and II. The high hERG1 expression in G1–G2 samples well agrees with its prevailing expression in intestinal type gastric cancers, which are usually well-differentiated tumors. Moreover, the fact that hERG1 is expressed in a significant percentage of TNM stages I and II, suggests that the overexpression of the channel is an early event during gastric cancer progression. This is different from what occurs in colorectal cancers (16, 17) and from what reported by Shao and colleagues (14) and Ding and colleagues (15) in gastric cancer. The latter discrepancy could be traced back to the fact that both studies were performed on Asian patients' cohorts, which have different clinico-pathological characteristics compared with non-Asian ones (30), and by the use of different antibodies and scoring systems. The significant early expression of hERG1 during gastric cancer progression shown by us, is further strengthened by the statistically significant interaction on OS between hERG1 expression and T. In particular, we showed that hERG1 has a negative prognostic impact in T1 patients, a finding that could be exploited for treatment stratification of gastric cancer patients. In fact, as a final goal, we demonstrated that hERG1 channels might represent a pharmacologic target. In particular, we showed that treatment of tumor-bearing mice with a specific hERG1 blocker (E4031) decreased both the tumor volume and intratumoral angiogenesis. Both parameters were even more inhibited when E4031 was added in combination with the VEGF-A antibody (bevacizumab; ref. 31), with a schedule that was able to maintain tumor inhibition even after treatment suspension. Therefore, the blocking of hERG1 through noncardiotoxic blockers (either existing, as in ref. 32, or under development; www.blackswanpharma.com) could be proposed as a combination treatment able to overcome the well-known resistance to antiangiogenesis treatments in solid cancers (33).

On the whole, our findings suggest the possibility of including hERG1 channels into biomolecular panels of gastric cancer prognostic markers, in the near future. Further studies are needed to validate hERG1 impact on clinical course or response to chemotherapy, to design a personalized treatment combining noncardiotoxic hERG1 blockers and antiangiogenesis drugs in hERG1-positive patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):
Study supervision: P. Bechi, A. Arcangeli, L. Boni

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