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

Olivia Crociani1, Elena Lastraioli1, Luca Boni3, Serena Pillozzi1, Maria Raffaella Romoli1, Massimo D’Amico1, Matteo Stefanini1, Silvia Crescioli1, Antonio Taddei2, Lapo Bencini4, Marco Bernini4, Marco Farsi4, Luca Saragoni11, Elisa Giommoni5, Silvia Gasperoni5, Francesco Di Costanzo5, Franco Roviello10, Stefania Beggelli6, Aldo Scarpa6, Luca Messerini1, Anna Tomezzoli8, Carla Vindigni9, Paolo Morgagni11, Giovanni De Manzoni7, Paolo Bechi2, and Annarosa Arcangeli1

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); 1–11. ©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 瞵-go-go gene (EAG) family (7). In particular, we demonstrated that K⁺ channels encoded by the human ether 瞵-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...
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.

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 (dithiothreitol).

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 $2 \times 10^5$ 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 hERG1 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 hIHERG 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 hERG1 expression in gastric cancer cells and primary samples. The relevance of posttranslational mechanisms was excluded, because no differences in the amount of the hERG1iso 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
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
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-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, Kvl 1.3 (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 O₂ 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...
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 clinico-pathological 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 caisic at 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).

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 mm³, 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 mm³. 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 1. Association between hERG1 expression and clinical and pathologic variables

<table>
<thead>
<tr>
<th>Variable</th>
<th>hERG1 positivity rate</th>
<th>OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>All cases</td>
<td>69.1%</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Age, y</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;70</td>
<td>69.3%</td>
<td>1 (ref.)</td>
<td>0.927</td>
</tr>
<tr>
<td>≥70</td>
<td>68.9%</td>
<td>0.98 (0.67–1.44)</td>
<td>0.64</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>69.1%</td>
<td>1 (ref.)</td>
<td>0.979</td>
</tr>
<tr>
<td>Female</td>
<td>69.0%</td>
<td>1.00 (0.67–1.47)</td>
<td>0.87</td>
</tr>
<tr>
<td>Site of primary tumor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antrum, cardias</td>
<td>64.8%</td>
<td>1 (ref.)</td>
<td>0.017</td>
</tr>
<tr>
<td>Body</td>
<td>68.7%</td>
<td>1.19 (0.75–1.88)</td>
<td>0.87</td>
</tr>
<tr>
<td>Fundus</td>
<td>80.4%</td>
<td>2.23 (1.30–3.82)</td>
<td>0.64</td>
</tr>
<tr>
<td>Gastric stump, linitis plastica</td>
<td>61.1%</td>
<td>0.85 (0.39–1.84)</td>
<td>0.51</td>
</tr>
<tr>
<td>TNM stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>75.3%</td>
<td>1 (ref.)</td>
<td>0.268</td>
</tr>
<tr>
<td>II</td>
<td>79.8%</td>
<td>1.30 (0.61–2.75)</td>
<td>0.64</td>
</tr>
<tr>
<td>III</td>
<td>65.0%</td>
<td>0.61 (0.33–1.14)</td>
<td>0.47</td>
</tr>
<tr>
<td>IV</td>
<td>65.3%</td>
<td>0.62 (0.33–1.17)</td>
<td>0.47</td>
</tr>
<tr>
<td>Pathologic grading</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1, G2</td>
<td>80.9%</td>
<td>1 (ref.)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>G3, G4</td>
<td>62.3%</td>
<td>0.39 (0.25–0.61)</td>
<td>0.031</td>
</tr>
<tr>
<td>Lauren type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intestinal</td>
<td>79.0%</td>
<td>1 (ref.)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diffuse</td>
<td>53.4%</td>
<td>0.30 (0.20–0.47)</td>
<td>0.979</td>
</tr>
<tr>
<td>Mixed</td>
<td>47.2%</td>
<td>0.24 (0.13–0.43)</td>
<td>0.979</td>
</tr>
<tr>
<td>VEGF-A status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>25.6%</td>
<td>1 (ref.)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Positive</td>
<td>75.2%</td>
<td>9.31 (3.61–24.0)</td>
<td>0.001</td>
</tr>
</tbody>
</table>
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

---

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

<table>
<thead>
<tr>
<th>Variable</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HR (95% CI)</td>
</tr>
<tr>
<td>Age, y</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;70</td>
<td>1 (ref.)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>≥70</td>
<td>1.63 (1.34–1.99)</td>
<td>0.001</td>
</tr>
<tr>
<td>Site of primary tumor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antrum, cardias</td>
<td>1 (ref.)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Body</td>
<td>1.14 (0.89–1.47)</td>
<td>1.03 (0.80–1.33)</td>
</tr>
<tr>
<td>Fundus</td>
<td>1.37 (1.06–1.76)</td>
<td>1.18 (0.90–1.54)</td>
</tr>
<tr>
<td>Gastric stump, linitis plastic</td>
<td>2.52 (1.67–3.80)</td>
<td>1.96 (1.28–3.00)</td>
</tr>
<tr>
<td>Pathological grading</td>
<td>1.14 (0.93–1.41)</td>
<td>0.77 (0.59–1.00)</td>
</tr>
<tr>
<td>Intestinal</td>
<td>1 (ref.)</td>
<td>0.207</td>
</tr>
<tr>
<td>Diffuse</td>
<td>1.55 (1.23–1.94)</td>
<td>1.44 (1.07–1.94)</td>
</tr>
<tr>
<td>Mixed</td>
<td>1.79 (1.30–2.45)</td>
<td>1.74 (1.20–2.51)</td>
</tr>
<tr>
<td>VEGF-A status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>1 (ref.)</td>
<td>0.510</td>
</tr>
<tr>
<td>Positive</td>
<td>1.00 (0.59–1.68)</td>
<td>0.91 (0.62–1.32)</td>
</tr>
<tr>
<td>hERG1 status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>1 (ref.)</td>
<td>0.726</td>
</tr>
<tr>
<td>Positive</td>
<td>0.96 (0.78–1.19)</td>
<td>1.22 (0.95–1.57)</td>
</tr>
</tbody>
</table>

Crociani et al.
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. S1).

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).
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 breast 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 Ca2+ 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 clinicopathological 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 outcome 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.

Authors’ Contributions
Conception and design: P. Bechi, A. Arcangeli
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Taddei, L. Bencini, M. Bernini, M. Farsi, A. Scarpia, A. Tomezzoli, C. Vindigni, P. Morgagni, L. Saragoni, E. Giommoni, F. Roviello, G. De Manzioni, S. Beghelli, F. Di Costanzo, S. Gasperoni, P. Bechi, S. Pillozzi, M. Stefani
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): O. Crociani, E. Lastraioli, L. Boni, P. Bechi, A. Arcangeli
Writing, review, and/or revision of the manuscript: O. Crociani, E. Lastraioli, L. Boni, P. Bechi, A. Arcangeli
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Study supervision: P. Bechi, A. Arcangeli, L. Boni

Acknowledgments
The authors thank Dr. L. Guasti for performing WB experiments on primary samples, E. Wanke and A. Becchetti for useful suggestions and manuscript revision.

Grant Support
This work was supported by Associazione Italiana per la Ricerca sul Cancro (AIRC, grant no. 1662), Association for International Cancer Research (AICR, grant no. 06-0491), Istituto Toscano Tumori (ITT; DD Regione Toscana No. 6888) to A. Arcangeli, Ente Cassa di Risparmio di Firenze to F. Di Costanzo, and Veneto Regional Grant (No. 6421) to A. Scarpia.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received September 24, 2013; revised November 27, 2013; accepted December 20, 2013; published OnlineFirst January 21, 2014.
References


27. Um SY, McDonald TV. Differential association between HERG and KCN1 or KCN2. PLoS ONE 2007;2:e933.


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

Olivia Crociani, Elena Lastraioi, Luca Boni, et al.

Clin Cancer Res  Published OnlineFirst January 21, 2014.

Updated version  Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-13-2633

Supplementary Material  Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2014/01/21/1078-0432.CCR-13-2633.DC1

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, use this link http://clincancerres.aacrjournals.org/content/early/2014/03/04/1078-0432.CCR-13-2633. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.