hERG1 channels regulate VEGF-A secretion in human gastric cancer: clinicopathological correlations and therapeutical implications.

Olivia Crociani1*, Elena Lastraio1*, Luca Boni2*, Serena Pillozzi1, Maria Raffaella Romoli1, Massimo D’Amico1, Matteo Stefanini1, Silvia Crescioli1, Antonio Taddei3, Lapo Bencini4, Marco Bernini4, Marco Farci4, Stefania Beghelli5, Aldo Scarpa5, Luca Messerini1, Anna Tomezzoli6, Carla Vindigni7, Paolo Morgagni8, Luca Saragoni8, Elisa Giommoni9, Silvia Gasperoni9, Francesco Di Costanzo9, Franco Roviello10, Giovanni De Manzoni11, Paolo Bechi10 and Annarosa Arcangeli1#§

1 Department of Clinical and Experimental Medicine, University of Florence, Italy
2 Clinical Trials Coordinating Center, Azienda Ospedaliero- Universitaria Careggi, Florence, Italy
3 Surgery and Translational Medicine, University of Florence, Italy
4 General Surgery and Surgical Oncology, Azienda Ospedaliero- Universitaria Careggi, Florence, Italy
6 Department of Pathology and Diagnostics, University of Verona, Italy
7 Pathology Division, Borgo Trento Hospital, Verona, Italy
8 Pathology Division, Azienda Ospedaliero-Universitaria Senese, Siena, Italy
9 General Surgery and Division of Pathology, Morgagni-Pierantoni Hospital, Forli, Italy
10 Medical Oncology, Azienda Ospedaliero-Universitaria Careggi, Florence, Italy
11 Department of General Surgery and Oncology, University of Siena, Italy
12 Division of Surgery, University of Verona, Italy

“On behalf of Gruppo Italiano di Ricerca Cancro Gastrico”
* equally contributed to the paper
# equally contributed to the paper
§ Correspondence to:
Annarosa Arcangeli, M.D., Ph.D.
Dipartimento di Medicina Sperimentale e Clinica
Viale G.B. Morgagni, 50
50134 Florence, Italy
Phone: +39 055 2751283
Fax: +39 055 2751281
e-mail: annarosa.arcangeli@unifi.it

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Conception and design: Arcangeli A, Crociani O, Lastraioli E, Boni L, Bechi P


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STATEMENT OF 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 centres. 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 GC progression and could identify high risk T1 stage patients. We also show that hERG1 regulates VEGF-A secretion in GC, and that a combined treatment of mice xenografted with GC cells with hERG1 blockers and anti-VEGF-A antibodies has an additive anti tumoral effect. Thus, there is the potential for a personalized treatment combining non cardiotoxic hERG1 blockers and anti-angiogenic drugs in hERG1 positive GC patients.
ABSTRACT

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

Experimental design: hERG1 expression was tested in a wide (508 samples) Italian cohort of surgically resected GC patients, by immunohistochemistry (IHC) and Real Time Quantitative- (RQ) PCR. The functional link between hERG1 and the Vascular Endothelial Growth Factor-A (VEGF-A) was studied in different GC cell lines. The effects of hERG1 and VEGF-A inhibition was 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 TNM 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 (HIF). Treatment of immunodeficient mice xenografted with human GC 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 GC since its early stages; (ii) drives an intracellular pathway leading to VEGF-A secretion; (iii) can be exploited to identify a GC patients’ group where a combined treatment with anti-angiogenic drugs and non cardiotoxic hERG1 inhibitors could be proposed.
INTRODUCTION

Despite the decrease in gastric cancer (GC) mortality observed worldwide in the last decades, GC 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 the present paper, hERG1 activity is modulated by hypoxia [9] and has an important role in 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 GC has been addressed by different groups. It was first shown that hERG1 channels are functionally expressed in GC 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 two small cohort retrospective studies. Despite these results, consistent evidences about hERG1 clinical significance in GC and its prognostic impact are still lacking.

Purpose of the present paper is to better analyze the expression of hERG1 channels, as well as its prognostic role in a wide Italian cohort of GC, with peculiar emphasis to its functional
correlation with VEGF-A. Moreover, the possible therapeutic effect of combining hERG1 and VEGF-A targeting treatments in GC 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 GC 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 [16]. All samples were divided into three aliquots, one immediately fixed in formalin, one frozen in liquid nitrogen for storage and the other stored in RNAlater® (Ambion; Austin, TX USA).

Moreover, a multicenter cohort of GC archival samples (n= 389) mainly assembled as Tissue Micro Arrays 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 pre-operative radiotherapy or chemotherapy were excluded. Considering both the prospective and the retrospective cohorts, 579 samples were analyzed. Diagnosis and histological grading were assessed using standard criteria by experienced pathologists (LM, AT, CV, and LS).

**Immunohistochemistry (IHC).** hERG1 and VEGF expression were retrospectively tested in 579 patients by IHC, performed as previously reported [17] using the antibodies reported in Table S1. Stained sections were analyzed as in [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 LB using SAS version 9.2 (SAS Institute, Cary, NC).

**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 (Table S2).
RNA extraction and Reverse Transcription (RT). Total RNA was extracted using Trizol® (Invitrogen; Carlsbad CA, USA), following the manufacturer’s protocol. RT was performed using 1μg of total RNA and Superscript II (Invitrogen; Carlsbad CA, USA), according to the manufacturer’s instructions but avoiding the use of reducing agents (dithiotreitol).

Real-Time Quantitative PCR (RQ-PCR). RNA extraction, reverse transcription and RQ-PCR were performed as in [11]. Further details are reported in Supplementary Data. The primer sequences are reported in 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 Table S3.

VEGF-A secretion. Cells were seeded into 24-well cell culture plates at 2 x 10⁵ cells/ml in standard culture medium. After 24 hours, the medium was removed and 0.5 ml of Optimem (Gibco; Carlsbad CA, USA) was added. After an additional 24hours incubation, the medium was collected and VEGF-A measured using the DuoSet ELISA Development System (R&D Systems; Wiesbaden, Germany). 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: 1) hERG1-specific inhibitors (E4031 or WAY 123,398 (WAY), at the final concentration 40 μM, as described in [10]); 2) the PI3K/Akt inhibitor LY294002 (10 μM, Sigma), or the PI3K/Akt inhibitor perifosine (20 μM, kindly provided by Dr. A. Martelli, University of Bologna).

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

HIF activity. HIF activity was measured employing cells transfected with the hypoxia responsive element-luciferase reporter gene vector, kindly provided by Dr. A Giaccia (Stanford University School of Medicine, Stanford, USA), 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 paper was approved by the Italian Ministry of Health (document n° 140/2009-B).
RESULTS

**Analysis of hERG1 expression in primary GC**

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

hERG1 protein expression was determined through IHC in GC 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 GC were negative to hERG1 staining (Fig. 1D). Panels E-H show the hERG1 staining in GC cases of different grading and stage, whereas low magnification pictures in which hERG1 focal expression can be better observed are in Fig S1. These data are discussed in the paragraph “*Clinical significance of hERG1 in GC*”. Western Blot (WB) experiments performed in some of the samples collected confirmed IHC data (Supplementary Data- 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 GC*) 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 two *hERG1* transcripts, *hERG1A* and *hERG1B* [19]. Fig. 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 GC samples. On the other hand, the hERG1B transcript was never expressed at levels comparable or higher than the normal mucosa (Fig. S3).

To gain insights on the genetic mechanisms underlying hERG1A over-expression in GC, we performed molecular analyses using different GC cell lines as a model. As shown in Fig. 2B, the hERG1A transcript was expressed in all the GC cell lines, although at variable levels, from more than one hundred folds (AKG cells) to nihil (AGS cells) (Fig. 2B). No expression of the hERG1B isoform was detected in any of the GC cell lines tested (not shown) [12]. These results were confirmed by WBs performed on membrane extracts (see Supplementary Data- 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, two of the four examined GC cell lines showed high hERG1 expression, with a percentage mimicking results obtained in GC primary samples.

We also analyzed pre- and post-translational mechanisms that could underlie the different hERG1A expression in GC cells and primary samples. The relevance of post-translational mechanisms was excluded, since no differences in the amount of the hERG1USO protein [20] (e.g. the main post-translational mechanism affecting hERG1 protein levels) were detected (Fig. S5). We then analyzed the methylation status in a subset of GC primary samples (n=13). To this purpose, 7 samples expressing (see asterisks in Fig. 2D) and 6 non-expressing the hERG1A transcript were analyzed, looking at two CpG islands, one located within the promoter and one adjacent to the transcription start site (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. On the other hand, 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 two CpG islands analyzed does not appear to explain the different hERG1A levels in GC 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 two 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 to AGS cells (Fig. 2E). Hence, an increased mRNA stability (witnessed by a slower rate of mRNA decay) could underlie the *hERG1A* over-expression in GC cell lines. This finding could be translated to GC primary samples.

**hERG1 channels drive VEGF-A secretion in GC.**

We then evaluated the functional role of hERG1 channels in GC cells. In particular, we analyzed whether a functional link between hERG1 and VEGF-A existed in GC. All the GC 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 (Tab 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. On the other hand, 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, three different anti-*hERG1* siRNAs (α-siRNAs 1-3) were tested, all effective in reducing *hERG1* expression (Tab 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 (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 due to off-target effects, since 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 (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 (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 two HIF-α transcripts: HIF-1α and HIF-2α [22]. Hence, we tested whether the same pathway was controlled by hERG1 in GC cells. We first determined the transcriptional activity of HIF in GC cells. HIF activity (measured as luciferase activity, see Supplementary Data) was decreased by either E4031 or WAY (Fig. 3D). On the other hand it increased after switching the cells to hypoxia, as expected. HIF activity was also measured quantifying the expression levels of HIF-1α− and HIF-2α-dependent genes. hERG1 inhibition decreased the expression of HIF-1α and HIF-2α co-regulated (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 two different PI3K/Akt inhibitors LY294002 (LY) and perifosine (Fig. 3D), which also significantly decreased VEGF-A secretion (Fig. S6).
measured both Akt activity (by an in vitro kinase assay using GSK-3 as a substrate Fig. 3G, left panel), and Akt phosphorylation (Fig. 3G, right panel): both were decreased by hERG1 inhibitors.

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

Clinical significance of hERG1 in GC.

hERG1 expression was then correlated with clinico-pathological parameters as well as with patients’ survival in the whole cohort of GC 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 Data- 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 pathological grade characterized the casistic under study (Table S6). Moreover, 63.8% of the samples were classified as Lauren’s 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 Fig. S7). For hERG1 expression analyses, data obtained with the hERG1 polyclonal antibody were used (representative pictures are reported in the Supplementary Data- Fig. S8, taking into account two 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’s intestinal type GC compared to the diffuse type (see also Fig. 1, panels C 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.1 panels E-H). Finally, a strong correlation
with VEGF-A emerged (p<0.001). Often the two proteins were co-expressed in the same tissue sample and, more specifically, in the same cancerous epithelial cells, with a similar pattern of expression (see Supplementary Data- Fig. S9).

After a median follow up of 11.1 years (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 pathological parameters (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 pharmacological targeting: in vivo experiments.**

Finally, we determined whether hERG1 channels could represent good targets for anti-neoplastic therapy in GC. To test this possibility, we analyzed immunodeficient, athymic nu/nu mice subcutaneously injected with hERG1-expressing GC 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 two weeks starting from the day after inoculum. The masses obtained were then analyzed five days after the suspension of treatment E4031 significantly decreased tumor growth, as evidenced by the decrease of the tumor volume (from 277.3 mm$^3$ 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 (panel “Control” on the right of Fig. 4C), while those within the masses from E4031-treated mice were
fewer although longer (panel “E4031” on the right of Fig. 4C), with a higher perivascular fibrosis (see arrow in right panel). The reduced vasculature of GC 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 two agents (Fig. 4D, left panel). After completing the treatment schedule, tumors started to grow again, except in the combined treatment regimen. In particular, when monitored after ten 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 which underwent the combined treatment (Fig. 4F).
DISCUSSION

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

hERG1 channels turned out to be over-expressed in both primary gastric tumors and GC 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, that 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, since KCNE2 behaves also as hERG1 accessory subunit [26].

The hERG1 expression we found in GC primary samples and cell lines confirms previous data [12, 13]. Moreover, we showed that hERG1 is over-expressed 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 (1) only the full length hERG1A transcript is over-expressed, a finding completely different from what occurs in other tumors, such as leukemias [11, 19], where only the hERG1B transcript is over-expressed. This suggests the existence of a tumor type-related hERG1 isoform signature; (2) hERG1A over-expression in GC correlates with an increased stability of the corresponding mRNA, in highly hERG1 expressing GC cells, a fact that candidates this as the mechanism underlying hERG1A over-expression in GC samples. Consistently, we excluded a significant contribution to hERG1 over-expression 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 over-expression of hERG1 in GC is witnessed by a strong immunostaining of GC samples. In this study, we used two 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 two 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 favoured the use of the polyclonal antibody, whose results well fitted with those obtained measuring hERG1A transcript levels by RQ-PCR (Fig. S11).

The functional role of hERG1 channels in GC was analyzed in GC cell lines and we provided evidence that hERG1 regulates VEGF-A transcription and hence VEGF-A secretion in GC. Hence, hERG1 function in GC is similar to that discovered in brain tumors [10] and during mouse colorectal carcinogenesis [27]. The regulation of VEGF-A secretion occurs exclusively in GC cells expressing hERG1 at high levels, a fact proven by both pharmacological 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 GC, 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 GC, hERG1 behaves as a cell cycle device, capable of regulating cell proliferation [12, 13], as well as as a progression-related gene, mainly involved in the regulation of tumor angiogenesis. While 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 GC, studying a large Italian cohort of 508 GC patients, encompassing different TNM stages. hERG1 expression strongly correlated with intestinal Lauren’s histological 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 GCs, 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 over-expression of the channel is an early event during GC progression. This is different from what occurs in colorectal cancers [16,17] and from what reported by Shao XD et al. [14] and Ding X-W et al. [15] in GC. 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 to non-Asian ones [30], and by the use of different antibodies and scoring systems. The significant early expression of hERG1 during GC 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 GC patients. In fact, as a final goal, we demonstrated that hERG1 channels might represent a pharmacological 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) [31], with a schedule which was able to maintain tumor inhibition even after treatment suspension. Therefore, the blocking of hERG1 through non cardiotoxic blockers (either existing, as in [32], or under development (www.blackswanpharma.com)) could be proposed as a combination treatment able to overcome the well known resistance to anti-angiogenesis treatments in solid cancers [33].
On the whole, our findings suggest the possibility of including hERG1 channels into biomolecular panels of GC prognostic markers, in the near future. Further studies are needed to validate hERG1 impact on clinical course or response to chemotherapy, in order to design a personalized treatment combining non cardiotoxic hERG1 blockers and anti-angiogenesis drugs in hERG1 positive patients.
FIGURE LEGENDS

Figure 1. hERG1 protein expression in primary GC samples.

IHC was performed on GC samples and paired healthy mucosa. A-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 GC samples of different TNM stage and Grading. Four representative microphotographs are reported, showing hERG1 expression (evaluated with polyclonal antibody) in samples classified as TNM IAand IV, G2 and G3 as indicated in the pictures. Magnification: 20x.

Figure 2. hERG1A characterization in primary GC and cell lines.

A) hERG1A transcript expression in GC primary samples. Graph shows data obtained by RQ-PCR analysis performed in 28 primary GC 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 GC cell lines. In the histogram, data obtained from all the experiments performed on different GC 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 (upper panels) and TSS CpG island (lower panels). 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 are means ± SEM of three separate experiments, each carried out in duplicate. **: p<0.02; ***: p<0.01 (Student’s T test).
Figure 3. Characterization of hERG1 expression and VEGF-A expression and secretion in GC cell lines: the effects of hERG1 inhibition or over-expression on VEGF-A secretion.

A) VEGF-A secretion in GC cell lines. In the histogram, data obtained from all the experiments performed on GC cell lines are summarized. B) The effect of hERG1 blocking on VEGF-A secretion in GC cells. Ion channel blockers were added 24 hours before VEGF-A measurement. hERG1 inhibitors were used as in [10]. Data from four different experiments, each carried out in duplicate, are reported as mean ± SEM. TEA data refer to two 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 pharmacological blocking. Hypoxic HIF-1α transcriptional activity was also shown as control. E) Fold induction of HIF(s) target genes after hERG1 pharmacological blocking, GLUT1, glucose transporter 1; ANGPTL-4, angiopoietin-like 4; LDHA, lactate dehydrogenase. F) HIF-2α dependent expression after hERG1 pharmacological blocking. G) Effects of the hERG1 blocker E4031 on Akt activity (left panel) and on Akt phosphorylation (right panel) 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 are means ± SEM of two or three separate experiments. * p<0.05 (Student’s T test). *: p<0.05; **: p<0.02; ***: p<0.01 (Student’s t test).

Figure 4. hERG1 channels in GC 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 mm³; 246.25 ± 0.095 mm³) and E4031-treated mice (black bar, 19.6 ± 14.5 mm³; 103.75 ± 0.05 mm³). Data are reported as mean of two experiments (four animals/group) ± SEM. Right panel: time course of tumor growth in control (▲) or E4031-treated mice (■). 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 [11] after staining with an anti-CD34 antibody and is reported as μm² 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) Histological 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 400x. 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. Panel D: time course of tumour masses growth in the four different groups. Treatment schedule is reported below. Panel 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 are reported as mean ±SEM. *: p<0.05; **: p<0.02; ***: p<0.01 (Student’s T test). Panel 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 are reported as mean ±SEM. *: p<0.05; **: p<0.02 (Student’s T test).
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REFERENCES


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**Table 1.** Association between hERG1 expression and clinical and pathological variables.
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Table 2. Univariate and multivariate evaluation of prognostic role for overall survival of clinical and pathological variables.
Fig. 2
Clinical Cancer Research

hERG1 channels regulate VEGF-A secretion in human gastric cancer: clinicopathological correlations and therapeutical implications.

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

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