Ovarian Cancer and Genetic Susceptibility: Association of A61G Polymorphism in the *EGF* Gene

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Growth factors play an essential role in regulating cellular proliferation, and lack of control is characteristic of malignant development. The epidermal growth factor (*EGF*) gene codifies a growth factor that binds to the EGF receptor (EGFR), which is involved in activating pathways that promote cellular proliferation, survival, migration and differentiation. The purpose of this study was to appraise the association between *EGF* gene A61G polymorphism with ovarian cancer susceptibility. A total of 564 DNA samples were analysed from 175 women with ovarian cancer and 389 women without cancer, through PCR-RFLP. We found a decreased risk for developing ovarian cancer in GG carriers compared to AA carriers (OR = 0.46, CI = 0.25–0.83, *P* = 0.010). The seemingly protective role in GG carriers was observed in women under 53 years of age (OR = 0.38, CI = 0.16–0.86, *P* = 0.011) and in patients diagnosed with advanced stage disease (OR = 0.38, CI = 0.18–0.81, *P* = 0.012). Allelic comparison evidenced similar results, with decreased risk for G allele. We further observed a linear trend for G allele in cancer risk. Moreover, we analysed the influence of genotypes in the time to onset of the disease and observed that GG carriers had ovarian cancer later than AA carriers (*P* = 0.035). We hypothesize that this polymorphism confers protection for ovarian cancer development. Exp Biol Med 234:241–245, 2009

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Introduction

Extracellular environment controls cell survival, and growth factors are one of the most important signallers in this process (1). Epidermal growth factor (EGF) is a key factor in promoting cell survival, and after binding to EGF receptor (EGFR) it causes activation of several signal transduction pathways (1), promoting cell proliferation, survival, migration and differentiation (2, 3).

Growth factors are important in initiating and maintaining neoplastic transformation (4). Tumour cells synthesize high levels of growth factors regulating their proliferation through autocrine and paracrine mechanisms (2, 4). EGFR and its ligands are often overexpressed in human carcinomas and they contribute to tumour progression through different mechanisms (2). EGFR and EGF-like peptides are involved in over 70% of all cancers (5).

The reproductive tract malignancies have important repercussion in women. Ovarian cancer is the sixth most common cancer and the seventh cause of death from cancer in women, with approximately 204,000 new cases and 125,000 deaths in 2002 (6). The normal ovary expresses EGFR but when compared with primary ovarian carcinomas, the expression in this disease shows significantly higher levels (7). It has also been reported that EGF regulates oocyte maturation and follicular growth (8), and it is involved in autonomous proliferation of ovarian carcinoma cells (7).

Shahbazi *et al.* (9) identified a polymorphism at position 61 of the *EGF* gene consisting of a substitution of G for A (A61G). This variation *in vitro* produced less quantity of EGF in cells from AA individuals than cells

Table 1. EGF Genotype and Allele Frequencies in Patients with Ovarian Cancer and in Healthy Controls

	Cases ($n = 175$) *n* (frequencies)	Controls ($n = 389$) *n* (frequencies)	OR	95% CI	*P*
Genotype[a]					
AA	67 (0.38)	120 (0.31)	1		
AG	83 (0.48)	180 (0.46)	0.83	0.55–1.25	0.344
GG	25 (0.14)	89 (0.23)	**0.50**[b]	0.28–0.89	**0.011**
Allele					
A	217 (0.62)	420 (0.54)	1		
G	133 (0.38)	358 (0.46)	**0.72**	0.55–0.94	**0.012**

[a] Analysis for linear trend according to the presence of null, one, or two G alleles: $P = 0.014$.
[b] Age adjusted risk for GG vs AA: OR = 0.46, 95% CI = 0.25–0.83, $P = 0.010$.

from GG or GA individuals and consequently the GG carriers were associated with an increased risk of developing malignant melanoma when compared with AA genotype ($P < 0.0001$) (9).

To the best of our knowledge, no reports have been published regarding the role of *EGF* polymorphism in ovarian cancer. The aim of our study was to assess the effect of A61G polymorphism in ovarian cancer susceptibility in the Portuguese population.

Materials and Methods

Subjects. This case-control study included 175 women (median 53 years of age) with histologically confirmed ovarian cancer, from the Portuguese Institute of Oncology-Porto between 1999 and 2001, according to other studies performed by our group (10, 11). Regarding the histological classification of tumours, 57% were serous, 15% clear cell, 14% mucinous, 10% endometrioid and 4% had other histological types. Patients were staged according to FIGO statements and were followed by the same medical oncologist. The cancer-free control women ($n = 389$, median 41 years of age) were recruited from the Blood Donors Bank Institute. Individuals from both groups were Caucasian and residents in the same geographic area and all of them gave consent according to the Helsinki Declaration.

EGF A61G Genotype Analysis. Genomic DNA was extracted from 8 ml peripheral blood obtained with a standard venipuncture technique using EDTA-containing tubes, and isolated from the white blood cell fraction of each sample, using a standard salting out protocol (12).

The *EGF* A61G genotypes were determined using the PCR-RFLP assay according to a previously published protocol by Shahbazi *et al*. (9). Two primers, forward: 5′-TGT CAC TAA AGG AAA GGA GGT-3′ and reverse: 5′-TTC ACA GAG TTT AAC AGC CC-3′, were used to amplify the 242 base pair (bp) fragment. PCR reactions were performed in a 50 µl reaction volume containing: 1x Taq Buffer, 1.5 mM of $MgCl_2$, 0.2 mM of dNTPs, 0.3 µM of each primer and 1U Taq DNA polymerase. Thermocycler parameters were as follows: 95°C for 5 mins; 35 cycles of 94°C for 60 s, 55°C for 60 s and 72°C for 60 s; and a final extension step at 72°C for 5 mins. PCR products were digested overnight at 37°C with 2 U of the restriction enzyme and 10 µl of PCR products to a final volume of 15 µl. The restriction fragments were then analyzed by electrophoresis in 3% (w/v) agarose gel stained with 0.5% ethidium bromide and photographed under UV illumination. The abrogation of the site recognized by the restriction enzyme *Alu*I, produces 4 fragments of 15, 34, 91 and 102 bp (A allele), while the G allele corresponds to 3 fragments of 15, 34 and 193 bp. In the gel only fragments of 91, 102 and 193 bp are visible.

Statistical Analysis. Chi-square test was used to compare categorical variables. Odds ratio (OR) and 95% confidence interval (95% CI) were calculated to assess the relationship between the polymorphism and ovarian cancer susceptibility, followed by age-adjusted logistic regression analysis. Linear trend analysis was performed to evaluate the risk for cancer according to the presence of zero, one, or two G alleles. Hardy-Weinberg equilibrium was tested by a Pearson goodness of fit test to compare the observed vs. the expected genotype frequencies.

The waiting time to onset of the disease (WTO) was defined as the interval between the time of initial exposure to the risk factor (A61G) and the time of disease onset. Thus, we calculated the cumulative probabilities for having disease by the Kaplan-Meier methodology and the primary analysis of time-to-event end points for WTO with the use of a two-sided log-rank test.

All statistical analysis in this study was performed with the computer software SPSS for Windows (version 15.0) and Epi Info (version 6.04), considering a statistical significant *P* value when $P < 0.05$.

Results

Genotype frequencies for both groups were: AA (0.38), AG (0.48) and GG (0.14) in ovarian cancer cases and AA (0.31), AG (0.46) and GG (0.23) for controls (Table1). Genotype frequencies for this polymorphism were in Hardy-Weinberg equilibrium for both groups (controls, $P = 0.996$; cases, $P = 0.400$). The homozygous G carriers presented lower risk for developing ovarian cancer in crude (OR = 0.50, 95% CI = 0.28–0.89, $P = 0.011$) and in age-adjusted logistic regression analysis (OR = 0.46, 95% CI = 0.25–0.83, $P = 0.010$) (Table 1). Linear trend analysis showed a

Table 2. Association of A61G Polymorphism with Clinicopathological Parameters in Patients with Ovarian Cancer Compared with Controls

	Cases *n* (frequencies)	Controls *n* (frequencies)	OR	95% CI	*P*
Age $\leq$ 53 ($n = 85$)					
Genotype[a]					
AA	35 (0.41)	84 (0.29)	1		
AG	40 (0.47)	138 (0.49)	0.70	0.40–1.22	0.177
GG	10 (0.12)	64 (0.22)	**0.38**	0.16–0.86	**0.011**
Allele					
A	110 (0.65)	306 (0.53)	1		
G	60 (0.35)	266 (0.47)	**0.63**	0.43–0.91	**0.009**
FIGO stage III/IV[b] ($n = 97$)					
Genotype[c]					
AA	40 (0.41)	120 (0.31)	1		
AG	46 (0.48)	180 (0.46)	0.77	0.46–1.28	0.280
GG	11 (0.11)	89 (0.23)	**0.37**[d]	0.17–0.80	**0.006**
Allele					
A	126 (0.65)	420 (0.54)	1		
G	68 (0.35)	358 (0.46)	**0.63**	0.45–0.89	**0.006**

[a] Analysis for linear trend according to the presence of null, one, or two G alleles: $P = 0.011$.
[b] Staging for cases only.
[c] Analysis for linear trend according to the presence of null, one, or two G alleles: $P = 0.007$.
[d] Age adjusted risk for GG *vs.* AA: OR = 0.38, 95% CI = 0.18–0.81, $P = 0.012$.

cumulative protective role effect for G allele in cancer susceptibility ($P = 0.014$).

Furthermore, women diagnosed before 53 years of age, who are simultaneously GG carriers, had protection for ovarian cancer (OR = 0.38, 95% CI = 0.16–0.86, $P = 0.011$) (Table 2), although no association was found for women over 53 years old (data not shown). In stratified analysis according to stage of disease, also GG carriers were protected for being diagnosed with advanced stage (III and IV) (OR = 0.38, 95% CI = 0.18–0.81, $P = 0.012$) (Table 2), albeit there was lack of association for earlier stages (data not shown).

Kaplan-Meier cumulative hazard function plots and log-rank test showed an earlier onset of disease for homozygous A carriers when compared to homozygous G carriers ($P = 0.035$) (Fig. 1). We observed a trend for G allele cumulative protective effect in the WTO when all three genotypes were analysed (AA, AG and GG), although no statistically significant difference was achieved (median: AA, 62 years; AG, 65 years; GG, 70 years; $P = 0.067$).

Discussion

Cancer predisposition may be due to the combination of low penetrance genetic variants. Polymorphisms may have higher significance to public health than individual risks associated with familiar cancer (13–15). Genetic association studies on the *EGF* A61G genetic polymorphism have already been performed in cancer, with controversial results. Nevertheless, considerable concordance exists in *in vitro* studies associating the *EGF* 61 G allele with EGF overexpression (9, 16–22).

In this case-control study, we analysed the association between a functional polymorphism (A61G) of the *EGF* gene and the risk for developing ovarian cancer. We found a protective effect for the development of ovarian cancer in homozygous G carriers (OR = 0.46, $P = 0.010$). The protective effect of GG carriers was also observed for women $\leq$53 years of age (OR = 0.38; $P = 0.011$) and for being diagnosed with advanced disease (stage III and IV) (OR = 0.38; $P = 0.012$). Furthermore, Kaplan-Meier curve

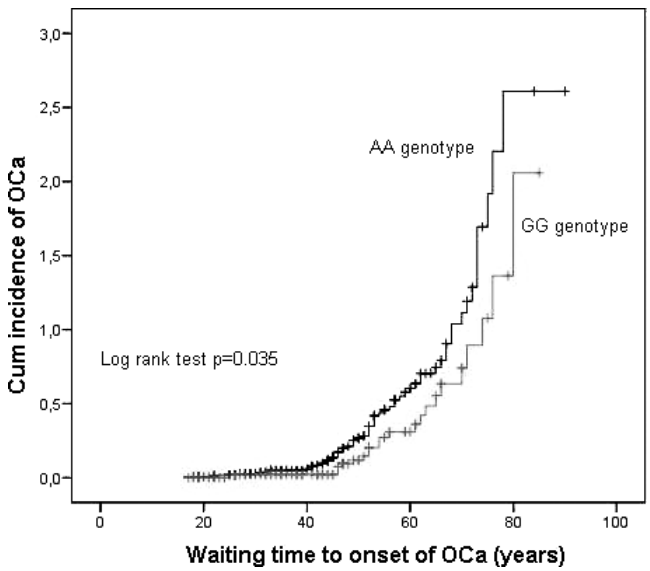

Figure. 1. Association between A61G polymorphism and the waiting time to onset of disease (WTO). Cumulative hazard function plots by the Kaplan-Meier methodology and log-rank test ($P = 0.035$).

analysis showed later onset of disease for GG genotype carriers, compared to AA ($P = 0.035$).

The EGF is a growth factor that activates a signal transduction pathway promoting proliferation, migration and differentiation (23); nevertheless, some authors showed that, after binding to its receptor, they are both internalized, sourcing the formation of endosomes and ultimately degradation (23, 24). The protective effect for the EGF overexpression in G allele carriers that we observed in this study may be explained by the increased bioavailability of EGF and the subsequence removed of EGFR from the cell surface, due to its degradation. In fact, other studies showed that EGFR expression on cell surface decreases abruptly when a certain concentration of EGF is added to cell lines (25, 26). Specific EGF ligands promote a long lasting effect, inhibiting EGFR recycling (26). A low availability of EGFR will decrease the EGF/EGFR pathway activation and eventually protect ovarian cancer development. Another recent study reports that in some cell lines, EGF paradoxically inhibits proliferation in high concentrations and induces loss of adhesion, cell cycle arrest and apoptosis (27).

It has been suggested that EGFR has pleiotropic cell responses (23) that are regulated by signals when numerous negative regulatory mechanisms act, such as: availability of the ligand to the receptor and terminal signal inactivation through receptor internalization and degradation (24). Furthermore, this regulation varies according to the arrangements of ligand-receptor engagement, tyrosine phosphorylation and subsequent receptor dimerization combinations, as well as, on the stage and context of cell type, and cell growth, different signalling transduction pathways can be activated (24). As active pathways are not known in each different tumour, we can explain our different results for this tumour type, because different regulatory mechanisms of EGFR may depend on tissue or tumour type.

It is known that EGF acts in the ovary and consequently EGFR expression is present since the functional initiation of this organ. When we analysed the genotype influence in the time to onset of disease, we observed that GG carriers developed cancer later than the homozygous A. It is known that germinative line genetic variants are part of the genetic background of individuals from *in utero* until death. This is even more relevant if we are in the presence of a functional polymorphism, as in the case of *EGF* A61G. In this case it will impact in the concentration of growth factor both locally and systemically, eventually leading to less EGFR in ovarian cells, and consequently lower activation of the EGF/EGFR cascade. We hypothesize that women who carry G homozygous genotype are more prone to later onset of disease (8 years later).

The protective effect of GG also occurred in individuals that have more advanced staging. Overexpression of EGFR is more frequent in the ovarian cancer staged at III and IV (Lassus *et al.,* 2002) (28), suggesting that this pathway is relevant for advanced ovarian cancer. Considering this evidence, we hypothesize that GG carriers, who have lower EGFR at cell surface, may have an increased protection for advanced stage ovarian cancer. Further studies on EGFR expression according to *EGF* A61G in ovarian tumours are required to confirm this hypothesis.

We also showed that GG carriers in younger women (<53 years) have lower risk for developing ovarian cancer. EGF is expressed in the ovary (29, 30) and acts in the evolution of ovarian follicles (31–33). It inhibits the expression of hormones produced in the ovary (estrogen and progesterone) and decreases of FSH (follicle-stimulating hormone) (34–37). Since younger women are more exposed to sexual hormones and these are associated with a greater cell proliferation, a decrease of these hormones promoted by higher levels of EGF in GG carriers reduces the risk for ovarian cancer in pre-menopausal women.

In conclusion the polymorphism A61G in ovarian cancer was found to confer protection. Protection was also found for being diagnosed with higher stage and in women younger than 53 years. The waiting time to onset of ovarian cancer was longer in GG carriers. These apparent paradoxal results involving *EGF* in ovarian cancer protection are supported by recent findings of EGFR internalization and degradation, which may be regulated through ligand availability. Further studies on this issue should be undertaken to analyse the role of EGF/EGFR pathway in ovarian cancer and the role of genetic background in this mechanism.

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1. Henson ES, Gibson SB. Surviving cell death through epidermal growth factor (EGF) signal transduction pathways: implications for cancer therapy. Cell Signal 18:2089–2097, 2006.
2. Normanno N, Bianco C, Strizzi L, Mancino M, Maiello MR, De Luca A, Caponigro F, Salomon DS. The ErbB receptors and their ligands in cancer: an overview. Curr Drug Targets 6:243–257, 2005.
3. Araújo A, Ribeiro R, Azevedo I, Coelho A, Soares M, Sousa B, Pinto D, Lopes C, Medeiros R, Scagliotti GV. Genetic polymorphisms of the epidermal growth factor and related receptor in non-small cell lung cancer—a review of the literature. Oncologist 12:201–210, 2007.
4. Ciardiello F, Tortora G. Interactions between the epidermal growth factor receptor and type I protein kinase A: biological significance and therapeutic implications. Clin Cancer Res 4:821–828, 1998.
5. Levitzki A. EGF receptor as a therapeutic target. Lung Cancer 41:S9–S14, 2003.
6. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin 55:74–108, 2005.
7. Salomon DS, Brandt R, Ciardiello F, Normanno N. Epidermal growth factor-related peptides and their receptors in human malignancies. Crit Rev Oncol Hematol 19:183–232, 1995.
8. Maruo T, Ladines-Llave CA, Samoto T, Matsuo H, Manalo AS, Ito H, Mochizuki M. Expression of epidermal growth factor and its receptor in the human ovary during follicular growth and regression. Endocrinology 132:924–931, 1993.
9. Shahbazi M, Pravica V, Nasreen N, Fakhoury H, Fryer AA, Strange

RC, Hutchinson PE, Osborne JE, Lear JT, Smith AG, Hutchinson IV. Association between functional polymorphism in EGF gene and malignant melanoma. Lancet 359:397–401, 2002.
10. Medeiros R, Pereira D, Afonso N, Palmeira C, Faleiro C, Afonso-Lopes C, Freitas-Silva M, Vasconcelos A, Costa S, Osório T, Lopes C. Platinum/paclitaxel-based chemotherapy in advanced ovarian carcinoma: glutathione S-transferase genetic polymorphisms as predictive biomarkers of disease outcome. Int J Clin Oncol 8:156–161, 2003.
11. Santos AM, Sousa H, Pinto D, Portela C, Pereira D, Catarino R, Duarte I, Lopes C, Medeiros R. Linking TP53 codon 72 and P21 nt590 genotypes to the development of cervical and ovarian cancer. Eur J Cancer 42:958–963, 2006.
12. Müllenbach R, Lagoda PJ, Welter C. An efficient salt-chloroform extraction of DNA from blood and tissues. Trends Genet 5:391, 1989.
13. Balmain A, Gray J, Ponder B. The genetics and genomics of cancer. Nat Genet 33:238–244, 2003.
14. Ponder BA. Cancer genetics. Nature 411:336–341, 2001.
15. Peto J. Cancer epidemiology in the last century and the next decade. Nature 411:390–395, 2001.
16. Rees J. Functional polymorphism in EGF gene and malignant melanoma. Lancet 360:570, 2002.
17. McCarron SL, Bateman AC, Theaker JM, Howell WM. EGF +61 gene polymorphism and susceptibility to and prognostic markers in cutaneous malignant melanoma. Int J Cancer 107:673–675, 2003.
18. Amend KL, Elder JT, Tomsho LP, Bonner JD, Johnson TM, Schwartz J, Berwick M, Gruber SB. EGF gene polymorphism and the risk of incident primary melanoma. Cancer Res 64:2668–2672, 2004.
19. Bhowmick DA, Zhuang Z, Wait SD, Weil RJ. A functional polymorphism in the EGF gene is found with increased frequency in glioblastoma multiforme patients and is associated with more aggressive disease. Cancer Res 64:1220–1223, 2004.
20. Goto Y, Ando T, Goto H, Hamajima N. No association between EGF gene polymorphism and gastric cancer. Cancer Epidemiol Biomarkers Prev 14:2454–2456, 2005.
21. Costa BM, Ferreira P, Costa S, Canedo P, Oliveira P, Silva A, Pardal F, Suriano G, Machado JC, Lopes JM, Reis RM. Association between functional EGF+61 polymorphism and glioma risk. Clin Cancer Res 13:2621–2626, 2007.
22. Ribeiro R, Soares A, Pinto D, Catarino R, Lopes C, Medeiros R. EGF genetic polymorphism is associated with clinical features but not malignant phenotype in neurofibromatosis type 1 patients. J Neurooncol 81:225–229, 2007.
23. Wells A. EGF receptor. Int J Biochem Cell Biol 31:637–643, 1999.
24. Singh AB, Harris RC. Autocrine, paracrine and juxtacrine signaling by EGFR ligands. Cell Signal 17:1183–1193, 2005.
25. Salazar G, Gonzalez A. Novel mechanism for regulation of epidermal growth factor receptor endocytosis revealed by protein kinase A inhibition. Mol Biol Cell 13:1677–1693, 2002.
26. Friedman LM, Rinon A, Schechter B, Lyass L, Lavi S, Bacus SS, Sela M, Yarden Y. Synergistic down-regulation of receptor tyrosine kinases by combinations of mAbs: implications for cancer immunotherapy. Proc Natl Acad Sci U S A 102:1915–1920, 2005.
27. Zhao X, Dai W, Zhu H, Zhang Y, Cao L, Ye Q, Lei P, Shen G. Epidermal growth factor (EGF) induces apoptosis in a transfected cell line expressing EGF receptor on its membrane. Cell Biol Int 30:653–658, 2006.
28. Lassus H, Sihto H, Leminen A, Joensuu H, Isola J, Nupponen NN, Butzow R. Gene amplification, mutation, and protein expression of EGFR and mutations of ERBB2 in serous ovarian carcinoma. J Mol Med 84:671–681, 2006.
29. Ozcakir HT, Giray SG, Ozbilgin MK, Uyar Y, Lacin S, Caglar H. Immunohistochemical detection of transforming growth factor-alpha, epidermal growth factor, and vascular endothelial growth factor expression in hyperstimulated rat ovary. Acta Obstet Gynecol Scand 84:887–893, 2005.
30. Leung PCK, Steele GL. Intracellular signaling in the gonads. Endocr Rev 13:476–498, 1992.
31. Marui T, Ladines-Llave CA, Samoto T, Matsuo H, Manalo AS, Ito H, Mochizuki M. Expression of epidermal growth factor and its receptor in the human ovary during follicular growth and regression. Endocrinology 132:924–931, 1993.
32. Ashkenazi H, Cao X, Motola S, Popliker M, Conti M, Tsafriri A. Epidermal growth factor family members: endogenous mediators of the ovulatory response. Endocrinology 146:77–84, 2005.
33. Conti M, Hsieh M, Park J-Y, Su Y-Q. Role of the epidermal growth factor network in ovarian follicles. Mol Endocrinol 20:715–723, 2006.
34. Hsueh AJ, Welsh TH, Jones PB. Inhibition of ovarian and testicular steroidogenesis by epidermal growth factor. Endocrinology 108:2002–2004, 1981.
35. Jones PBC, Welsh TH, Hsueh AJW. Regulation of ovarian progestin production by epidermal growth factor in cultured rat granulosa cells. J Biol Chem 257:11268–11273, 1982.
36. Pulley DD, Marrone BL. Inhibitory action of epidermal growth factor on progesterone biosynthesis in hen granulosa cells during short term culture: two sites of action. Endocrinology 118:2284–2291, 1986.
37. Findlay JK, Drummond AE. Regulation of the FSH receptor in the ovary. Trends Endocrinol Metab 10:183–188, 1999.