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Haemochromatosis gene (*HFE*) mutations in viral-associated neoplasia: Linkage to cervical cancer

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Abstract

The present study examines the frequency of the two main *HFE* mutations (C282Y and H63D) in a randomly selected population of 346 individuals including 201 DNA samples from women with cervical neoplasia (including high-grade squamous intraepithelial lesions and invasive squamous cell carcinoma) and a control population of 146 women from the same geographical area. We found a significantly lower risk of development of cervical neoplasia in H63D carriers (OR = 0.56; 95% CI 0.35–0.92; $p = 0.01$). Multivariate logistic regression analysis confirms this observation (OR = 0.55; 95% CI 0.35–0.88, $p = 0.01$). Regarding the C282Y mutation no association was found (OR = 1.32; 95% CI 0.53–3.33; $p = 0.52$). In addition, a significant difference between H63D carrier and non-carrier women on the time-to-onset of cervical lesions was observed (log-rank test: $p = 0.0012$). These results indicate that *HFE* could be considered a candidate modifier gene of viral-related neoplasia such as cervical carcinoma possibly by a dual role on iron metabolism and immunological system.
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Keywords: C282Y; Cervical carcinoma; H63D; HFE; HPV; Viral neoplasia

Epidemiological and molecular biology studies have demonstrated that human papillomavirus (HPV) is associated with the development of cervical carcinoma, a sexually transmitted disease [1–5]. High-risk HPV types (e.g., HPV16 and HPV18) are present in virtually all invasive squamous cell carcinomas of the uterine cervix [1,2,6–8]. HPV is a necessary but not sufficient element for the development of the aetiology of cervical cancer. Other risk co-factors of cervical cancer have been reported, such as carcinogen exposure, parity, certain nutritional alterations, and the genetic background of the host (e.g., HLA, NAT2, p53, and TNFα) [9–20].

Iron status is also thought to be involved in the aetiopathology of cancer [21]. Malignant cells have the capacity to express high numbers of transferrin receptors (TfR1 and TfR2) [22]. Hereditary haemochromatosis (HH) is an inherited metabolic defect of iron metabolism consisting of continued iron absorption in face of adequate iron stores resulting in parenchymal iron overload and eventually organ damage [23–26]. The HH gene, *HFE*, has two common missense mutations: C282Y and H63D [27]. Several studies have found that the most frequent missense *HFE* mutations (C282Y and H63D) may be associated with increased risk of cancer but controversial reports have also been published [28–37].

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HFE is localised on chromosome 6 (6p21.3), 4 Mb telomeric to the HLA-A locus and in spite of the large physical distance between the two genes, the two main mutations are in linkage disequilibrium with particular HLA-A alleles [38,39]. HLA polymorphisms have been extensively studied in cervical neoplasia with some, but not always, reproducible results [40–45]. However, to the best of our knowledge, the question of the frequency of the *HFE* mutations has not been studied in cervical carcinoma.

The main objective of the present work was to determine the frequency of the C282Y and the H63D mutations in *HFE* in cervical cancer, a virus-related neoplasia.

Subjects and methods

Study population. A total of 201 DNA samples extracted from peripheral blood of randomly selected Caucasian women with cervical disease from the north of Portugal were included in this study. The women included in this study had been consecutively diagnosed and treated at the Portuguese Institute for Oncology (IPO) Francisco Gentil in Oporto, Portugal, between 1998 and 2004. The average age at diagnosis of cervical neoplasia group was 48 ± 12 years (range 21–83 years; median age 47 years). The hospital-based collection of samples was approved by the Committee of Portuguese Government-Health Ministry (*Projectos de Investigação aplicada na Área de Cuidados de Saúde*).

Control population. The *HFE* allele frequencies of the study population were compared with allele frequencies in an apparently healthy control population ($n = 146$) of women from the same geographical area [46]. The average age at the time of *HFE* genotyping of the control group was 45 ± 13 years (range 18–88 years; median age 45 years). All individuals had given informed consent prior to their inclusion in the study.

DNA extraction and HFE genotyping. DNA was extracted according to standard procedures [47]. *HFE* genotyping was performed using a commercial kit, the Haemochromatosis StripASSAY^{B} (Vienna Lab, Austria), and by Denaturing High Performance Liquid Chromatography Analysis (DHPLC).

Briefly, the Haemochromatosis StripASSAY^{B} is based on the reverse-hybridisation principle. The *HFE* exon 2 and 4 gene sequences are simultaneously in vitro amplified and biotin-labelled in a single multiplex amplification reaction. Finally, the amplification products are selectively hybridised to a test strip, which contains oligonucleotide probes (wild type and mutant specific) immobilised as parallel lines. Bound biotinylated sequences are detected using streptavidin–alkaline phosphatase and colour substrates.

In the case of the DHPLC, 100–200 ng of genomic DNA was used to PCR-amplify an amplicon containing the two different *HFE* regions containing the H63D (in exon 2, 249 bp) and the C282Y (in exon 4, 268 bp), using a specific primer pair for each specific mutation (Ex2F2 5′-T GC ACT ACC TCT TCA TGG GTG-3′ and R2 5′-TGC TGT GGT TGT GAT TTT CCA-3′; and Ex4F2: 5′ CTC CTT TGG TGA AGG TGA CAC ATC-3′ and R2 5′-ATC ACA ATG AGG GGC TGA TCC A-3′ for the H63D and the C282Y, respectively) at a final concentration of 1 μM, 250 μM dNTPs (Fermentas) and 1.75 U of Expand High Fidelity PCR System (ROCHE).

For DHPLC analysis, either 10 μl of each sample alone or 5 μl of each sample mixed (1:1) with a control was heat-denatured at 95 °C for 5 min and slowly cooled to 25 °C for 45 min in a T Gradient Thermocycler (Biometra), to allow for the formation of heteroduplexes. Analysis was carried out using the 3500A WAVE DNA Fragment Analysis System (Transgenomic). Seven microlitres of each PCR product was loaded on a reversed-phase DNASep column (Transgenomic) preheated at 61.3 °C, in the case of exon 2, and 61.6 °C in the case of exon 4. In the case of exon 2 (H63D), hetero- and homoduplexes were loaded with 52.0% Buffer A (0.1 mol/L triethylamine acetate, pH 7.0) and 48.0% Buffer B (0.1 mol/L triethylamine acetate, pH 7.0, containing 25% (v/v) acetonitrile) and eluted with a linear acetonitrile gradient of Buffer A (Start 47.0%; Stop 38.0%) and Buffer B (Start 53.0; Stop 62.0), for 4.5 min, at a constant flow rate of 0.9 ml/min. In the case of exon 4 (C282Y), hetero- and homoduplexes were loaded with 51.3% Buffer A (0.1 mol/L triethylamine acetate, pH 7.0) and 48.7% Buffer B (0.1 mol/L triethylamine acetate, pH 7.0, containing 25% (v/v) acetonitrile) and eluted with a linear acetonitrile gradient of Buffer A (Start 46.3%; Stop 37.3%) and Buffer B (Start 53.7%; Stop 62.7%), for 4.5 min, at a constant flow rate of 0.9 ml/min. Prior to each set of injections, the column was equilibrated at starting conditions for 3 min and two blank (no-DNA) injections were administered, in order to guarantee for the maximum resolution in polymorphism genotyping.

Clinical staging. The clinical staging of the cervical neoplasia was established according to the TNM system (T, primary tumour; N, regional lymph nodes; M, distant metastasis) [48]. The study population was grouped as follows: (a) non-invasive lesions: 44 women with high-grade squamous intraepithelial lesion (H-SIL); (b) invasive cervical carcinoma (ICC): 41 women in stage T1, 93 women in stage T2, 21 in stage T3, and 2 in stage T4.

Statistical analysis. Analysis of data was performed using the computer software SPSS for Windows (SPSS, Chicago, IL; version 12.0) and Epi-Info (version 6.04). In all tests, the statistical significance was two-sided and was considered to be significant at $p < 0.05$.

In a first step, the allelic frequencies of the C282Y and H63D mutations were calculated in the study and control populations. In addition, the allelic frequencies of the C282Y and H63D mutations were compared within the different clinical stages. The statistical differences between distribution and allele frequencies in case patients and control subjects were identified by χ^2 test for significance. When the expected cell value in a cell was less than five, the Fisher exact test was used.

In a second step, odds ratio (OR) and 95% of confidence intervals (CI) were calculated to determine the magnitude and the statistical significance of the associations [49] between patients and controls.

In a third step, was considered the basic question: "Assuming that all H-SIL/ICC cases are infected with oncogenic HPV, what is the probability that women will experience the onset of disease before the age of X, in the presence of co-factors, supposing they survive so long?" [50]. To address this question it was hypothesised that co-factors associated to the genetic background may alter the time-to-onset for cervical disease (TTO) in those cases. The cumulative probabilities (cumulative hazard function plots) for having H-SIL/ICC were estimated by the Kaplan–Meier methodology [51]. The primary analysis of time-to-event end points for TTO was performed with the use of a two-sided log-rank test at the 5 percent level of significance.

Results

HFE genotypes and mutation allelic frequencies in cervical neoplasia (Table 1)

The distribution of the *HFE* genotypes and the corresponding allelic frequencies of the C282Y and H63D mutations among patients with cervical neoplasia, grouped according to clinical staging (H-SIL and ICC), and controls is summarised in Table 1.

In the cervical neoplasia group, the C282Y and H63D allelic frequencies found were 0.040 and 0.142, respectively. No statistically significant differences were observed in the C282Y allelic frequency between the case patients (0.040) and the controls (0.034). In the case of H63D mutation, the allelic frequency observed in women with cervical neoplasia (0.142) was significantly lower than in the control women (0.209, $p = 0.020$).

Table 1
Distribution of the *HFE* genotypes and allele frequencies among patients with cervical neoplasia and controls (total: 347 individuals)

	n	*HFE* genotypes						Allele frequencies	
		C282Y/C282Y *n* (%)	C282Y/Wt *n* (%)	C282Y/H63D *n* (%)	H63D/Wt *n* (%)	H63D/H63D *n* (%)	Wt/Wt *n* (%)	C282Y	H63D
Controls	146	1 (0.68)	5 (3.42)	3 (2.05)	46 (31.51)	6 (4.11)	85 (58.22)	0.034	0.209
Cervical neoplasia	201	0 (0)	14 (6.97)	2 (1.00)	43 (21.39)	6 (2.99)	136 (67.66)	0.040	0.142
H-SIL	43	0 (0)	2 (4.65)	0 (0)	7 (16.28)	1 (2.33)	33 (76.74)	0.023	0.105
ICC	158	0 (0)	12 (7.59)	2 (1.27)	36 (22.78)	5 (3.16)	103 (65.19)	0.044	0.152

H-SIL, high-grade squamous intraepithelial lesion; ICC, invasive cervical carcinoma.

Table 2
Odds ratio (OR) of the C282Y and H63D *HFE* mutations among cervical neoplasia patients and controls

	C282Y mutation				H63D mutation			
	Number of carriers/total number of subjects (%)	OR	95% CI	$p^{\#}$	Number of carriers/total number of subjects (%)	OR	95% CI	$p^{\#}$
Controls	9/146 (6.2)	1.00	Reference		55/146 (37.7%)	1.00	Reference	
Cervical neoplasia	16/201 (8.0)	1.32	0.53–3.33	0.52	51/201 (25.4%)	0.56	0.35–0.92	0.01
H-SIL	2/43 (4.7)	0.74	0.11–3.91	0.71	8/43 (18.6%)	0.38	0.15–0.93	0.02
ICC	14/43 (8.9)	1.48	0.58–3.85	0.37	43/158 (27.2%)	0.62	0.37–1.03	0.05

H-SIL, high-grade squamous intraepithelial lesion; ICC, invasive cervical carcinoma; OR, odds ratio; CI, confidence interval.
[#] Significance level of comparisons of the C282Y and H63D carrier frequencies between cervical carcinoma patients and controls using χ^2 test.

Carrier frequencies of the HFE mutations according to the cervical lesion (Table 2)

The carrier frequencies of the C282Y and the H63D *HFE* mutations were compared between cervical neoplasia patients and control subjects, globally and according to the clinical staging (H-SIL and ICC). In addition, OR and 95% of confidence interval (CI) for the H63D and C282Y mutation carriers were also calculated in order to estimate the risk of cervical neoplasia (Table 2).

No significant differences were found in the frequency of the C282Y mutation carriers between the global patient population and controls, and between the two major clinical stages of cervical neoplasia (H-SIL and ICC) and controls, as shown in Table 2, suggesting that this particular mutation is not associated with the risk for cervical carcinoma (Table 2).

Significant differences between patients and controls were observed however in women carrying the H63D variant (Table 2). The H63D carriers have significantly lower risk (OR = 0.56, 95% CI 0.35–0.92, $p = 0.01$) of cervical neoplasia than non-carriers. Multivariate logistic regression analysis confirms this observation in H63D carriers (OR = 0.55; 95% CI 0.35–0.88, $p = 0.01$). A lower risk was observed (OR = 0.38; 95% CI 0.15–0.93, $p = 0.02$), when the analysis was restricted to patients with H-SIL (Table 2). These results were also confirmed when the analysis takes into account the H63D *HFE* allele frequencies ($p = 0.020$ for cervical lesions and $p = 0.029$ for H-SIL).

Influence of C282Y and H63D HFE mutations on the age of onset of cervical lesions

In order to see if the *HFE* gene mutations have an influence on the age of onset of cervical neoplasia, were estimated the cumulative probabilities for the age of onset (time-to-onset, TTO) of cervical lesions by the Kaplan–Meier methodology (see Materials and methods). A significant difference (log-rank test: $p = 0.0012$) between H63D carrier and non-carrier subjects on the TTO of cervical lesions was observed. The median TTO for the H63D carriers was 60 years (95% CI 55–65) and 52 years (95% CI 49–55) in non-H63D carriers (Fig. 1). No statistically significant differences ($p = 0.9731$) were observed for median age of onset of cervical lesions between C282Y carriers and C282Y non-carriers (Fig. 2).

Discussion

To the best of our knowledge, the present study shows for the first time that a particular mutation in the *HFE* gene, H63D, has a carrier frequency significantly ($p = 0.01$) lower in women with cervical neoplasia when compared to the control population from the same geographical area, suggesting that H63D carriers have less probability of developing cervical cancer (OR = 0.53). Furthermore, when the cumulative probabilities for the age of onset (time-to-onset, TTO) of cervical lesions were estimated by the Kaplan–Meier methodology, H63D carriers present a late TTO, in comparison with non-carriers ($p = 0.0012$). Thus, we may hypothesise that H63D muta-

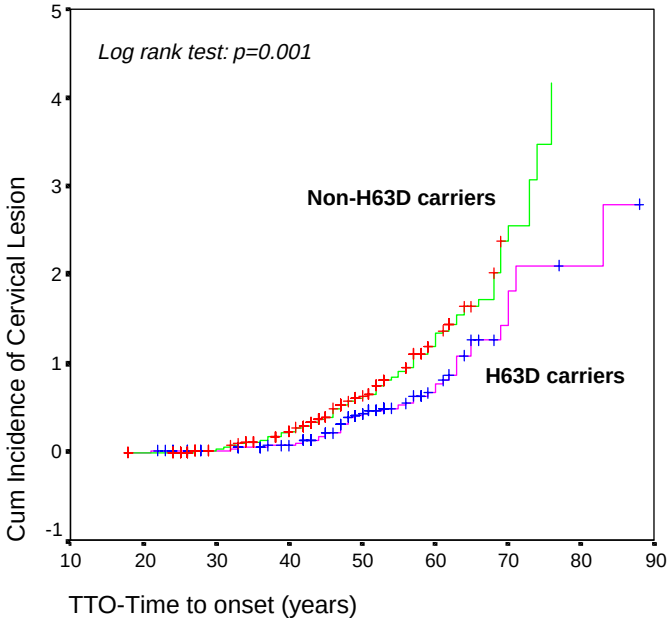

Fig. 1. Influence of the H63D *HFE* mutation in the time-to-onset (TTO) of the cervical lesions. Cumulative hazard function plots by the Kaplan–Meier methodology and log-rank test ($p = 0.001$).

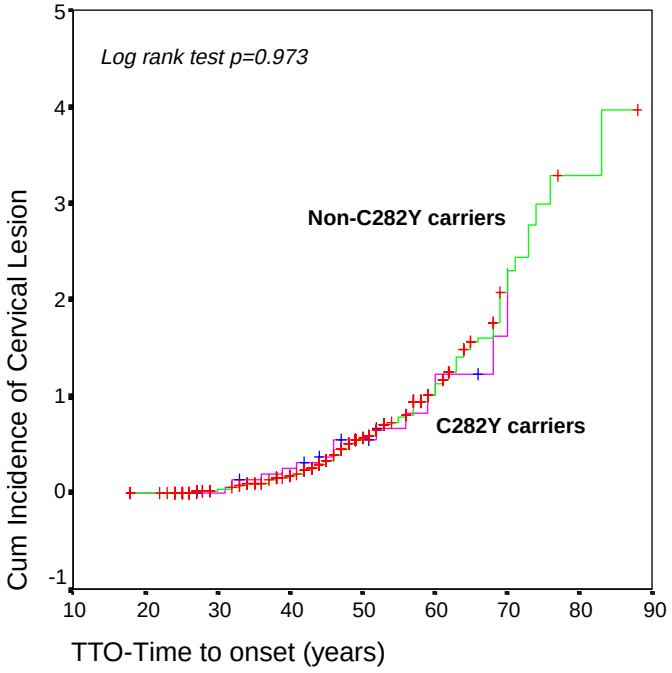

Fig. 2. Influence of the C282Y *HFE* mutation in the time-to-onset (TTO) of the cervical lesions. Cumulative hazard function plots by the Kaplan–Meier methodology and log-rank test ($p = 0.973$).

tion could have a protective role in the susceptibility to HPV infection and/or immunosurveillance of the infected cells of cervical neoplasia.

A possible explanation for the present findings could be linked to the putative impact of *HFE* on the immunological response. Previously published results have shown that the presence of the H63D mutation in carriers of the HLA-A29 allele was associated with higher numbers of $CD8^+$ T cells [39]. These results led the authors to postulate that a possible explanation for the polymorphic frequency of H63D could be related to the capacity of H63D carriers to respond successfully and thus survive life-threatening viral epidemics. In spite of the H63D substitution alone not preventing the HFE protein from reaching the cell surface [52], studies in human and mice show that the H63D mutation leads to moderate hepatic iron loading suggesting a partial loss of HFE function [53–55].

An effective immune surveillance against both HPV infection and HPV-associated neoplasia is an interplay between two important elements: the MHC-class I peptide presentation and the effective T-cell response. The influence of T-cell mediated response has been explored in several studies [56–64]. In addition, in cervical carcinomas, abnormalities in the MHC class I surface expression are frequently found constituting a potential strategy of malignant cells to escape the $CD8^+$ cytotoxic T-cell response [65–73]. Therefore, our results may be explained by the emerging role of *HFE* in immunological system and could be related to T-cell-mediated responses and/or MHC class I expression [74–76].

In summary, our results indicate that *HFE* could be considered a candidate modifier gene of viral-related neoplasia, such as cervical carcinoma. The definition of a genetic profile of cervical cancer may help to understand the dual role of HFE on iron metabolism and immunological system and its association with cancer development.

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