

ORIGINAL ARTICLE

Common filaggrin gene mutations and risk of cervical cancer

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ABSTRACT

Background. As carriers of filaggrin gene (*FLG*) mutations may have a compromised cervical mucosal barrier against human papillomavirus infection, our primary objective was to study their risk of cervical cancer.

Methods. We genotyped 586 cervical cancer patients for the two most common *FLG* mutations, R501X and 2282del4, using blood from the Copenhagen Hospital Biobank, Denmark. Controls (n = 8050) were genotyped in previous population-based studies. Information on cervical cancer, mortality and emigration were obtained from national registers. Odds ratios (OR) were estimated by logistic regression with adjustment for age at blood sampling, and weighted by the genotype-specific inverse probability of death between diagnosis and sampling. Hazard ratios (HR) were estimated by Cox regression with time since diagnosis as underlying time, and with adjustment for age at diagnosis and stratification by cancer stage.

Results. The primary results showed that *FLG* mutations were not associated with the risk of cervical cancer (6.3% of cases and 7.7% of controls were carriers; OR adjusted 0.81, 95% CI 0.57–1.14; OR adjusted+ weighted 0.96, 95% CI 0.58–1.57). Among cases, *FLG* mutations increased mortality due to cervical cancer (HR 4.55, 95% CI 1.70–12.2), however, the association was reduced after stratification by cancer stage (HR 2.53, 95% CI 0.84–7.59).

Conclusion. Carriage of *FLG* mutations was not associated with the risk of cervical cancer.

Filaggrin proteins are essential for the formation and function of the skin but are also expressed in oral and cervical mucosa. The proteins are known to align intracellular filaments leading to flattening of surface cell keratinocytes (squamous cells) and the degradation products maintain hydration, low pH, and provide immune modulation [1]. The impact of filaggrin deficiency on skin diseases was recognized in 2006, when two loss-of-function mutations in the filaggrin

gene (*FLG*), causing reduced expression of filaggrin, were discovered and shown to occur in 8–10% of North European and 5% of Asian study populations. The mutations were detected in almost all patients with the disease ichthyosis vulgaris – a semidominantly inherited disorder characterized by xerosis and palmar hyperlinearity – and were further strongly associated with atopic dermatitis [2,3]. *FLG* mutations are the first strong genetic risk factor yet

identified in the concept of complex diseases, which also encompass cervical cancer, and the first strong risk factor for atopic dermatitis [4,5].

In the 1980s, the expression of filaggrin in epithelia from cervical and oral biopsies was studied for its predictive value for premalignant or malignant squamous lesion [6–12]. In particular, filaggrin expression was found to be reduced in cervical lesions infected with the carcinogenic human papillomavirus (HPV) in a dose-dependent manner [6–8]. Currently, the role of filaggrin in the cervix remains unclear. We hypothesize that in a filaggrin-reduced cervix, HPV may have increased access and persistence into its target cells, the basal cells, for example due to reduced cervical acidity [13,14], compromised adherence between cells in the upper layers, or ongoing infection [15]. Results of epidemiological studies are compatible with this suspicion. For example, atopic dermatitis, in which up to 40% of patients carry *FLG* mutations, has been associated with higher occurrence of skin infections with wart viruses (HPV, *Molluscum contagiosum*), herpes simplex, herpes zoster, and *Staphylococcus aureus* [16–18]. A few of these studies had information on the filaggrin genotype and showed a higher prevalence of skin infections in mutation carriers with atopic dermatitis [19,20]. Furthermore, a study of two longitudinal cohorts showed an increased risk of cervical cancer in women who had atopic dermatitis and common warts in childhood [21]. As the role of *FLG* mutations in the etiology of cervical cancer has not been studied previously, we took advantage of the national biobank and patient registers in Denmark to genotype blood for the two most common *FLG* mutations, R501X and 2282del4, and examine the risk of cervical cancer in *FLG* mutation carriers.

Methods

Study population

Cases and controls were nested in the general population in the capital region in Denmark. Supplementary Table I (available online at <http://informahealthcare.com/doi/abs/10.3109/0284186X.2014.973613>) shows the study flow. We identified 808 in- or outpatients with a discharge diagnosis of cervical cancer and a blood sample in the Copenhagen Hospital Biobank by person-linkage between the National Patient Registry and the National Biobank Register (www.biobankdenmark.dk) using as link the Civil Registration System (CRS) number assigned to all Danish residents [22]. A total of 778 cases were genotyped; the remaining had no genotype call. The study included 586 cases whose first discharge diagnosis of cervical cancer verified in the

Danish Cancer Register updated until 2011 (denoted ‘verified cases’). The remaining cases were not considered eligible for inclusion, either because the discharge diagnosis was *before* the update but had never been verified (81 patients), or *after* the update and verification and also mortality was therefore yet unknown (110 patients). Controls included 8050 genotyped females from four previous population-based studies, excluding 25 females who developed cervical cancer after the blood sample was drawn.

Cervical cancer

Potential cases of cervical cancer were identified in the National Patient Register updated until May 2013 (ICD-8, code 180; ICD-10, code DC53) [23]. From the Danish Cancer Register updated until 2011 we obtained information on all cancer, morphology, and stage for all women in Denmark with cervical cancer including the studied cases (ICD-7, code 171; ICD-10, code DC53) [24,25]. Vital status and cause-specific mortality was obtained from the Civil Registration System and the Danish Register of Causes of Death updated until 2011 (Death from cervical cancer: ICD-8, code 180; ICD-10, code DC53) [22,26].

Control cohorts

Controls participated in four studies described elsewhere: ‘MONICA10’, 1,291 females [27]; ‘Inter99’, 3081 females [28]; ‘Health 2006’, 1848 females [29]; a study of the genetics of preterm delivery, 1855 females [30,31]. Briefly, the first three studies were general health studies of randomly selected adult men and women in the capital region in 1983–2008 [32] and the last study was a study including non-complicated pregnancies with preterm (cases) or term (controls) delivery and was nested among pregnant women who enrolled in 1996–2002 in the large Danish National Birth Cohort study (see also Supplementary Table I available online at <http://informahealthcare.com/doi/abs/10.3109/0284186X.2014.973613>) [33].

Copenhagen Hospital Biobank

The Copenhagen Hospital Biobank was established at the Department of Clinical Immunology at Copenhagen University Hospital, Rigshospitalet, to facilitate easier and standardized access to samples for medical research. Since February 2009, the Copenhagen Hospital Biobank has stored EDTA whole blood in excess from patient samples submitted for blood typing at the Copenhagen University Hospital, Rigshospitalet. Since February 2012,

Copenhagen Hospital Biobank stored similar sample excess from all other hospitals in the capital region (except from Copenhagen University Hospital Bornholm and psychiatric hospitals). Patients were included only once, i.e. repeated samples from the same patient were not included. At inclusion, May 2013, the Copenhagen Hospital Biobank contained more than 100 000 samples.

Filaggrin gene (FLG) mutations

For both cases and controls, we obtained information on the two most common null mutations in the *FLG* among Northern European descendants (GenBank NM_002016.1; c.1537C>T and c.2318_2321del; located on chromosome 1q21), commonly designated R501X and 2282del4, respectively. For cases and DNBC controls, DNA was genotyped with KASP™ technology (LGC Genomics Ltd, Herts, UK), whereas DNA from remaining controls were genotyped previously by Multiplex analysis (Department of Clinical Biochemistry at Copenhagen University Hospital, Gentofte, Denmark) [34]. Both methods uses a 3' anchored reverse primer to target and distinguish *FLG* mutations due to their sequence homology. As heterozygous carriers still produce filaggrin on the competent allele, we categorized zygosity: We defined carriers as homozygous when having the same mutation on both alleles, heterozygous when having one mutation only, and compound heterozygous when having different mutations on the two alleles (grouped as homozygous).

Ethics

The study was approved by the Danish Data Protection Agency (j. nr. 2008-54-0472), the steering committee of the Copenhagen Hospital Biobank, and the Ethical Committees of the Capital Region (j.nr. H-1-2013-055). Based on §10 of the Ethical committee law, dispensation for obtaining informed consent was granted because the studied blood samples were obtained as part of treatment and stored in a research biobank, all data were treated anonymous, results do not occur at the individual level, results of analyses have no consequences for the individual participant, the Danish law on handling of personal information is kept, and participants registered in the Danish Tissue Application Register are excluded.

Statistical analyses

The potential association of *FLG* mutations with the risk of cervical cancer was analyzed by comparing the occurrence of *FLG* mutations in cases and controls using an odds ratio (OR) estimated in a logistic

regression analysis with adjustment for age at blood sampling (one-year categories). In an additional analysis, the logistic regression was performed as a weighted analysis weighted by the genotype-specific inverse probability of survival between diagnoses and sampling. Survival estimates used in the weights were calculated using the Kaplan Meier estimator based on follow-up the first year. ORs for subgroups of cases were estimated by comparing the selected cases with all controls. The potential association of prognosis with *FLG* mutations among cervical cancer patients was analyzed by comparing mortality rates in patients with and without *FLG* mutations using a hazard ratio (HR) estimated in a Cox regression with time since diagnosis as underlying time, and with adjustment for age at diagnosis (continuous) and stratification by cancer stage (1, 2, 3, 4, localized, regional). Cervical cancer cases were followed from time of diagnosis of cervical cancer or sampling date, whichever came later (i.e. delayed entry), until end of 2011 or date of death from cervical cancer or other cause, whichever came first. Maximum likelihood estimation of ORs and HRs was performed using the PROC GENMOD and PROC PHREG procedure in SAS, version 9.4 software (SAS Institut, Inc., Cary, NC, USA). 95% CI for odds ratios were based on Wald's approximation except for the weighted logistic regression were bootstrapping with 5000 replications was used. Stage-specific ORs were compared by multinomial regression using PROC NLMIXED in SAS where a trend test was performed by assigning the values 1, 2, 3, and 4 to cases with stages 1–4 and treating stage as a continuous variable. Cancer characteristics for the cases in the study and all cervical cancer cases in Denmark were compared by a χ^2 -test, except survival after diagnosis which was compared in a Kaplan-Meier plot.

Results

Table I shows the characteristics of the 586 cases and 8050 controls. Overall, age at blood sampling was comparable despite the different years of blood sampling (cases, 2009–2013; controls, 1993–2008). However, older cases and younger controls were slightly more frequent (Table I), and two of four control cohorts were sampled with 5- and 10-year age intervals (data not shown). Most cases had blood sampled after being diagnosed with cervical cancer (7 months to 43 years, 53%; 2–6 months, 10%; < 2 months, 28%) whereas few cases were sampled before (1%). Cases were comparable with all cervical cancer patients in Denmark with regards to stage, metastasis, and mortality (see Supplementary Table II available online at <http://informahealthcare.com/doi/abs/10.3109/0284186X.2014.973613>, and Figure 1).

Table I. Characteristics of 586 cases of cervical cancer diagnosed 1967–2011 and 8050 controls from the capital region in Denmark.

	Cases		Controls	
	n	(%)	n	(%)
Year of blood sampling				
1993–2008	0	(0)	8075	(100)
2009–2013	586	(100)	0	(0)
Age at blood sampling (years)				
10–19	0	(0)	13	(0)
20–29	37	(6)	1103	(14)
30–39	103	(18)	1665	(21)
40–49	114	(19)	2103	(26)
50–59	91	(16)	1944	(24)
60–69	127	(22)	961	(12)
70–79	67	(11)	286	(4)
80–89	41	(7)	0	(0)
90–99	7	(1)	0	(0)

Risk of cervical cancer

Table II shows the odds ratio of cervical cancer according to filaggrin genotype. *FLG* mutations were not associated with cervical cancer, neither for heterozygous nor the few homozygous carriers. The overall risk estimate was slightly reduced (crude OR 0.81, 95% CI 0.57–1.12). Adjusting for age at blood sampling did not change the estimate substantially (OR 0.73, 95% CI 0.51–1.05). As most cases had blood sampled after diagnosis, we obtained information on mortality after diagnosis, and weighted the OR for the genotype-specific inverse probability of survival between date of diagnosis and date of blood sampling. This method yielded an estimate close to unity (age-adjusted and weighted OR 0.96, 95% CI 0.58–1.57).

We examined possible reasons for the observed lack of association between *FLG* mutations and cervical cancer. However, neither residual confounding by age due to extremes of sampling age nor the different genotyping methods used appeared to influence results (Supplementary Table III, available online at <http://informahealthcare.com/doi/abs/10.3109/0284186X.2014.973613>). We also examined cancer characteristics, and observed that the OR estimate of the association between *FLG* mutations and cervical cancer tended to increase with increasing

cancer stage at diagnosis (stage 1–4: ORs 0.61, 0.51, 1.04, and 1.81, respectively), however, the trend was not significant ($p = 0.07$) (Supplementary Table IV available online at <http://informahealthcare.com/doi/abs/10.3109/0284186X.2014.973613>). The estimate was not modified by having another site of cancer before cervical cancer (other genitalia, OR 0.97, 95% CI 0.12–7.80; not in genitalia, OR 0.67, 95% CI 0.16–2.86) or not having a strictly epithelial cervical cancer, i.e. adenocarcinoma (OR 0.66, 95% CI 0.26–1.64) or adenosquamous carcinoma (OR 1.43, 95% CI 0.42–4.89) but not squamous cell carcinoma (Supplementary Table IV available online at <http://informahealthcare.com/doi/abs/10.3109/0284186X.2014.973613>). Finally, in a sensitivity analyses, we included all the 778 genotyped cervical cancer patients we identified in the Copenhagen Hospital Bank based on discharge diagnoses (see study flow diagram in Supplementary Table I available online at <http://informahealthcare.com/doi/abs/10.3109/0284186X.2014.973613>), and also found no association (OR 0.74, 95% CI 0.53–1.02).

Prognosis of cervical cancer

As *FLG* mutations appeared to be associated with survival between diagnosis and sampling (i.e. see weighting of ORs in the first paragraph of last section), we examined an association with cause-specific case mortality (Table III). We observed that the rate of death caused by cervical cancer was higher among *FLG* mutation carriers when compared to wildtype carriers (HR 4.55, 95% CI 1.70–12.2), whereas this was not the case for other causes of death (HR 1.33, 95% CI 0.27–6.57). However, the association was attenuated after stratification by cancer stage (HR 2.53, 95% CI 0.84–7.59), and was based on five deaths from cervical cancer and two from other causes among *FLG* mutation carriers (Table III). Figure 1 shows survival time since cervical cancer diagnosis, and illustrates that the former five deaths took place in the first year after diagnosis, and that death among other cases were comparable with death among all women with cervical cancer in Denmark.

Table II. Odds ratio (OR) of cervical cancer according to filaggrin genotype (R501X/2282del4, wildtype) when comparing cases and controls from the capital region in Denmark.

Genotype	Cases (%)	Controls (%)	Crude OR (95% CI)	Adjusted for blood sampling age OR (95% CI)
Wildtype	549 (93.7)	7430 (92.3)	1 (ref.)	1 (ref.)
1 or both mutations	37 (6.3)	620 (7.7)	0.81 (0.57–1.14)	0.74 (0.51–1.06)
Heterozygous	35 (6.0)	597 (7.4)	0.79 (0.56–1.13)	0.74 (0.51–1.07)
Homozygous*	2 (0.3)	23 (0.3)	1.18 (0.28–5.00)	0.81 (0.17–3.88)

CI, confidence intervals.

*Includes compound heterozygous (0 cases and 5 controls).

Table III. Hazard ratio (HR) of death after cervical cancer according to filaggrin genotype (R501X /2282del4, wildtype) among cases sampled for genotyping blood until 2011,* capital region, Denmark.

Genotype	Cervical cancer cases n	Follow-up years	Dead n	Crude HR (95% CI)	Adjusted for age** HR (95% CI)	Adjusted for age** and stratification by stage HR (95% CI)
Death from cervical cancer						
Wildtype	391	494	42	1 (ref.)	1 (ref.)	1 (ref.)
Mutation	37	10	5	4.09 (1.58–10.62)	4.55 (1.70–12.2)	2.53 (0.84–7.59)
Death from other cause						
Wildtype	391	494	55	1 (ref.)	1 (ref.)	1 (ref.)
Mutation	37	10	2	0.95 (0.19–4.65)	1.33 (0.27–6.57)	1.03 (0.19–5.75)

CI, confidence intervals.

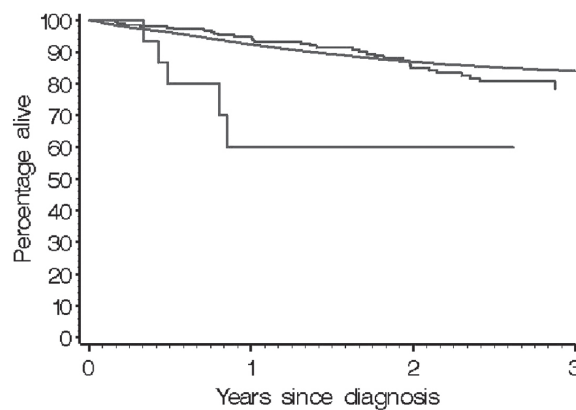
*158 of 586 cases were sampled 2012–2013 and were excluded because information on cause-specific mortality was not available; **Age at diagnosis.

Discussion

In this large case-control study, carriage of *FLG* mutations, and thus a possible impaired cervical mucosa barrier function, was not associated with an increased risk of cervical cancer as hypothesized. Our finding of a worse prognosis in cervical cancer patients carrying *FLG* mutations is interesting but should be interpreted with caution due to the possibility of an association with both severe cervical cancer and a worse prognosis.

We suspected that a filaggrin-reduced cervix increases HPV-access and -persistence and thereby the risk of cervical cancer. Although this hypothesis is not supported by the primary observation of a lack of association of *FLG* mutations with risk of cervical cancer, we believe the association with a worse prognosis lend support to the hypothesis, and is of interest because HPV-persistence has an important role

in the pathogenesis of cervical cancer [35–40]. Specifically, we observed that cases with *FLG* mutations had a four-fold higher rate of dying from cervical cancer than cases without *FLG* mutations. One explanation could be that mutation carriers more often develop severe cervical cancer, which in turn increases the risk of death. Another explanation could be that mutation carriers are poor responders to treatment, e.g. radiation therapy or chemotherapy. Our results suggest the former explanation is more likely, because the four-fold increase in the rate of death from cervical cancer attenuated after stratification for cancer stage and severe cancer stages more often comprised mutation carriers. Both of these findings were insignificant, showing that the statistical power for these extra analyses was limited. We recently observed that *FLG* mutations were associated with increased risk of all HPV-related pre-cancer



Years(x-axis)	0	0.5	1	1.5	2	2.5	3
FLG mutations	2	11	6	5	3	1	0
Wildtype	27	219	177	137	103	62	27
Total	29	229	183	142	106	63	27

Figure 1. Genotypic-specific and general survival after cervical cancer, Denmark. Survival since diagnosis of cervical cancer is shown for cases in the study sampled for genotyping blood until 2011 [red (lower) curve, *FLG* mutation carriers; blue curve, wildtype carriers], and for all cases in Denmark (green curve). The latter was the expected survival estimated based on all cases of cervical cancer in Denmark using direct-adjusted survival with age at diagnosis as covariate. The lower table shows number of studied cases at risk of death at selected time points during follow-up.

and cancer among the present control populations including men, albeit with too few cervical cancer cases [41]. Overall, we believe these findings should be interpreted with caution.

The large number of cases allowed us to study several cancer characteristics in detail using information from the Danish Cancer Registry. Among others we examined whether cases had another cancer before cervical cancer. If cancer initially did not start in the cervix, e.g. in the lungs where filaggrin is not expressed, then we would not expect an association with *FLG* mutations. Although most cases did not have a previous cancer, results were the same when excluding those who had. We also studied the morphological type of cancer, and suspected *FLG* mutations would not be associated with cervical cancer that develops in the endocervical epithelia (i.e. adenocarcinomas) because filaggrin is not normally expressed there but only in the exocervical epithelia where squamous cell carcinomas develop [7]. Regardless of cancer morphology, however, results were the same.

A characteristic of cases was the identification in a hospital biobank storing excess from blood typings and antibody screenings; cases were therefore likely in for an operation. It might be argued that this could influence the generalizability of our findings. However, our comparison with all cervix cancer patients in Denmark suggested that cases were representative in terms of metastasis and had only minor differences in cancer stage distributions (see Supplementary Table II, available online at <http://informahealthcare.com/doi/abs/10.3109/0284186X.2014.973613>). Interestingly, our findings were not materially different when including all the 778 cervical cancer patients we initially genotyped (to gain the largest possible statistical power notwithstanding case verification). Finally, cases and controls were comparable for important epidemiological factors, i.e. blood sampling age and sex, and although they were from different birth years the frequency of *FLG* mutation carriers are comparable across birth years [42].

Overall, the study had several advantages, including its large size and statistical power, the use of cancer diagnosis verified in the Danish Cancer Registry, the use of population-based controls, and the comparability with cervical cancer in Denmark for cancer stage, metastasis, and mortality after diagnosis (see Supplementary Table II available online at <http://informahealthcare.com/doi/abs/10.3109/0284186X.2014.973613> and Figure 1). In addition, the study also to some extent reproduced known associations with *FLG* mutations, e.g. with atopic dermatitis (see Supplementary Table V, available online at <http://informahealthcare.com/doi/abs/10.3109/0284186X.2014.973613>) [2,43].

In conclusion, carriage of common *FLG* mutations was not associated with the development of cervical cancer.

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Supplementary material available online

Supplementary Tables I–IV available online at <http://informahealthcare.com/doi/abs/10.3109/0284186X.2014.973613>