TITLE PAGE

Title: *Regulatory CDH4 genetic variants associate with risk to develop capecitabine-induced hand-foot syndrome*

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This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1002/CPT.2013](https://doi.org/10.1002/CPT.2013)

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Conflict of interest: All authors declared no competing interests for this work.

Funding

This study was supported by funds from ISCIII project grant PI12/00226 and by the Spanish Association against Cancer (AECC: Asociación Española contra el Cáncer). Human Genotyping lab is a member of CeGen, PRB3 and is supported by grant PT17/0019, of the PE I+D+i 2013-2016, funded by ISCIII and ERDF. Additional support comes from the Spanish Ministry of Economy and FEDER funds (grant BFU2013-48481-R to AL). S.R. is a predoctoral fellow

supported by the Severo Ochoa Excellence Program (Project SEV-2011-0191), and by the AECC. C.D was supported by AECC. A.K. is a fellow of the La Caixa International PhD Program.

Keywords

Capecitabine, hand-foot syndrome, *CDH4*, R-cadherin, skin barrier defects

ABSTRACT

Capecitabine-Induced Hand-Foot Syndrome (CiHFS) is a common dermatological adverse reaction affecting around 30% of capecitabine-treated cancer patients, and the main cause of dose reductions and chemotherapy delays. To identify novel genetic factors associated with CiHFS in cancer patients, we carried out an extreme-phenotype genome-wide association study (GWAS) in 166 breast and colorectal capecitabine-treated cancer patients with replication in a second cohort of 85 patients. We discovered and replicated a cluster of four highly correlated SNPs associated with susceptibility to CiHFS at 20q13.33 locus (top hit=rs6129058, HR=2.40, 95%CI =1.78–3.20; $P=1.2\times 10^{-8}$). Using circular chromosome conformation capture sequencing, we identified a chromatin contact between the locus containing the risk alleles and the promoter of *CDH4*, located 90 kilobases away. The risk haplotype was associated with decreased levels of *CDH4* mRNA and the protein it encodes, R-cadherin (RCAD), which mainly localizes in the granular layer of the epidermis. In human keratinocytes, *CDH4* downregulation resulted in reduced expression of involucrin, a protein of the cornified envelope, an essential structure for skin barrier function. Immunohistochemical analyses revealed that skin from patients with severe CiHFS exhibited low levels of RCAD and involucrin before capecitabine treatment. Our results uncover a novel mechanism underlying individual genetic susceptibility to CiHFS with implications for clinically relevant risk prediction.

MANUSCRIPT TEXT

INTRODUCTION

Capecitabine is a 5-fluorouracil prodrug frequently used for the treatment of breast and colorectal carcinomas¹. Approximately 30% of capecitabine-treated

patients develop an adverse skin reaction known as hand-foot syndrome, which is the most frequent cause of dose reduction or therapy discontinuation^{2,3}.

Factors associated with the appearance of capecitabine-induced hand-foot syndrome (CiHFS) include gender and age^{4,5}; genetic susceptibility variants involved in the metabolic biotransformation of capecitabine^{6–12} have also been identified. Although these factors provide important biological and clinical information, they do not fully explain the whole spectrum of the interindividual variabilities observed in CiHFS.

To identify additional CiHFS susceptibility variants, we conducted a two-stage extreme-phenotype genome-wide association study (GWAS) and addressed the functional significance of the replicated variants for CiHFS susceptibility.

MATERIALS AND METHODS

Patients

Capecitabine-treated breast and colorectal cancer patients were recruited prospectively through several Hospital Oncology Units across Spain. Blood samples were collected before first cycle of capecitabine and DNA was extracted in each unit according to their own protocols. To ensure accurate patient phenotyping and maximize genotyping efficiency we applied extreme phenotype sampling¹⁴. Case patients selected for both discovery and replication studies (n=88 and n=24, respectively) were required to have experienced grade 3 toxicity across any treatment cycle before completing eighteen cycles of treatment (over one year), and control patients (n=78 and n=61, in discovery and replication cohort respectively) were those not developing CiHFS during treatment (grade 0). CiHFS grade was determined according to the National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events (CTCAE) v 4.0¹⁵. All protocols were approved by the institutional review boards at each institution, and all subjects provided written informed consent.

Cumulative dose was calculated as the amount in mg/m² of capecitabine taken prior to the development of toxicity or at the end of follow-up, whichever was lower. Capecitabine was administered according to different schedules. Colorectal cancer patients were treated with a standard regimen (1250 mg/m² orally every 12 h on days 1-14, every 3 weeks), while breast cancer patients were treated either with the same standard regimen or with a continuous

regimen (800 mg/m² orally every 12 h daily during 21 days). Information on age at diagnosis, sex, primary tumor, treatment regimen, cumulative capecitabine dose and CiHFS grade was obtained from medical records.

Genotyping and quality control (QC)

DNA samples from the discovery cohort were genotyped using Illumina Human610-Quad BeadChips containing 616,795 SNPs according to the manufacturer's protocols. DNA samples from the replication cohort were genotyped by KASPar (Kbioscience) or Taqman (Thermo) assays and fluorescence was determined by the 7900HT Sequence Detection System (Applied Biosystems). Fine-mapping genotyping was performed by using the MassARRAY SNP genotyping system (Sequenom) and Infinium assay (Illumina) following the manufacturers' instructions. Details regarding QC are provided in Supplementary Materials and Methods (Figure S1).

Statistical analysis

Quantitative variables in discovery and validation samples were compared between cases and controls by means of Student's t-test or the Mann–Whitney U-test (for normally and not normally distributed data, respectively). Comparisons between categorical variables were performed using Fisher's exact test. All analyses were two-tailed and p-values < 0.05 were considered significant.

Primary analyses were based on Cox proportional hazards regression, considering the cumulative dose of capecitabine (mg/m²) up to the development of grade 3 CiHFS as the time metric. Patients not experiencing CiHFS of any grade during capecitabine treatment (controls) were censored at the total cumulative dose received. Estimated hazard ratios (HRs) were interpreted as relative risks of developing severe CiHFS (grade 3) at a given cumulative dose *versus* not experiencing CiHFS. Cox regression analysis was performed using the global data set to identify clinical factors associated with CiHFS development. Associations were assessed under an additive genetic model by stepwise multivariable analysis, adjusting for associated clinical characteristics. Cox regression analyses were performed using the R statistical environment (v.3.0.1) and PLINK (v.1.07)¹⁶. Kaplan-Meier comparisons of cumulative dose of

capecitabine taken up to the development of grade 3 CiHFS according to SNP genotype were performed using the SPSS software (v.18) and the log-rank test was applied.

To evaluate the possibility of false positives due to multiple testing, we used the Bayesian false discovery probability (BFDP) (Supplementary Materials and Methods, Figure S2).

Block definition, SNP selection and imputation

Details regarding block definition, SNP selection and imputation are provided in Supplementary Materials and Methods.

Cell lines and tissue samples

Cell lines: HaCaT cells (spontaneously immortalized human keratinocytes) were provided by Manuel Serrano (Spanish National Cancer Research Centre, CNIO, Spain) and cultured in MEME-based (M8167, Sigma) LC medium (Nowak and Fuchs) containing 0.05mM Ca^{2+} . Normal Human Epidermal Keratinocytes (NHEK) were purchased from Lonza (Cat N° 00192627; Lot N° 0000246915) and grown in Lonza's KGM-Gold media (Cat N° 00192060). To induce keratinocyte differentiation, 2mM Ca^{2+} was added to the medium. Cell lines were maintained under standard conditions and routinely tested for Mycoplasma.

Tissue samples: 44 healthy liver tissue samples were obtained from the CNIO (Spain) Tumor Bank. The study was approved by the ethics committee of the CNIO (Spain).

In silico analysis of transcription factor binding sites

To assess the potential for each of the identified variants to affect the binding of transcription factors we used two *in silico* tools: HaploReg v4.1^{17,18} and SNP2TFBS¹⁹.

Circular chromosome conformation capture (4C)-sequencing

NHEK and HaCaT keratinocytes homozygous for the reference and the risk haplotypes, respectively, were treated with 2mM Ca^{2+} for 72h before collection. Preparation of 4C samples was performed as previously described²⁰ with some modifications detailed in Supplementary Materials and Methods.

3C validation by PCR, real time-PCR and Sanger sequencing

NHEK and HaCaT 4C products digested with HindIII/DpnII and HindIII/CviQI were used for 3C validation. Details regarding primer design, PCR conditions

and the sequencing and quantification of PCR products are provided in Supplementary Materials and Methods.

mRNA expression analysis in healthy human liver tissues

Total RNA was extracted from cryopreserved liver tissues using 1ml TRIzol (Invitrogen) and concentration was determined by Nanodrop ND-1000 (Wilmington). Reverse transcription was performed in a single tube using 1 µg of total RNA, Superscript II Reverse Transcriptase (Invitrogen) and random hexamer primers following the manufacturer's instructions. *CDH4* mRNA was quantified by real-time PCR (RT-PCR) with the ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems), and fluorescent probes from the Human Universal ProbeLibrary Set (Roche). The expression levels of PCR products were normalized according to *ACTB* gene expression (Supplementary Materials and Methods, Table S1).

RCAD and involucrin protein expression

Normal paraffin-embedded skin samples derived from 16 breast cancer patients before capecitabine treatment (12 CiHFS grade 3 samples and 4 CiHFS grade 0 samples) were available for tissue microarray (TMA) construction. Details regarding TMA construction, immunohistochemistry, immunofluorescence, antibodies and quantification of RCAD and involucrin protein expression are provided in Supplementary Materials and Methods.

RCAD expression knock-down

For knock-down experiments HaCaT cells were transfected with the shRNA-expressing pGFP-V-RS plasmid (TR30007, OriGene) 12h after plating using X-tremeGENE 9 DNA transfection reagent (Roche) according to the manufacturer's instructions. Twenty-four hours after transfection 2mM Ca²⁺ was added to the medium for 48h. Control cells were transfected with a scrambled shRNA expressing plasmid. Details regarding RCAD shRNA sequences, antibodies used, RNA isolation, RT-PCR and immunoblotting are provided in Supplementary Materials and Methods.

RESULTS

Patient characteristics

Patients with high-grade toxicity (grade 3) were selected as cases, and those with no sign of CiHFS over the duration of their capecitabine treatment were

selected as controls. The discovery cohort and validation cohorts consisted of 166 and 85 patients, respectively, recruited at different hospital from Spain and a clinical trial comparing standard versus continuous administration of capecitabine in metastatic breast cancer¹³ (Table 1). The clinical characteristics of the discovery and replication cohorts are shown in Table 1. Age at diagnosis, gender, cumulative capecitabine dose, type of treatment and primary site of the tumor were found associated with a higher rate of CiHFS.

GWAS and fine-mapping

We carried out single-SNP associations using Cox regression analyses, considering the cumulative dose of capecitabine required for the development of grade 3 CiHFS as the time metric and adjusting for gender, tumor type and treatment regimen as selected in the stepwise multivariate model. Although no single SNP in the discovery phase reached the level of genome-wide significance ($P < 10^{-8}$), we selected those variants associated with a P -value $< 10^{-5}$ and those found to be noteworthy after applying Bayesian false discovery probability (BFDP) tests²¹ (BFDP < 0.8) ending up with a total of 11 SNPs for further replication (Figure S3; Table S3). Those variants were genotyped in the replication cohort ($n=85$ patients) and associations with grade 3 CiHFS were assessed. None of the already known genetic variants strongly associated with risk to develop CiHFS (rs12132152 and rs76387818 within *DPYD* gene¹²) achieve the threshold established to be replicated in our independent cohort. Cox regression analysis in replication cohort identified the intergenic SNP rs6093063 associated with CiHFS grade 3 (Hazard Ratio (HR): 2.27 (95% confidence interval (CI)=1.20–4.30, $P=0.011$). Although the p -value significance in the replication does not reach the Bonferroni adjusted threshold ($P=0.0045$), this result suggests a possible role of this variant on CiHFS phenotype. The combined analysis of the 245 subjects from both cohorts gave an HR of 2.17 (95%CI=1.63–2.88, $P=1.3 \times 10^{-7}$, Table S3, Figure S4). SNP rs6093063 is located in a linkage disequilibrium (LD) block of 38,501 bps spanning chromosome 20 positions 59,707,264–59,745,765 (GRCh37/hg19) at the 20q13.33 locus. In order to fine-map the rs6093063 association with CiHFS, we subsequently genotyped 34 SNPs and imputed 80 additional variants in the 245 subjects from both cohorts and tested their associations with CiHFS. Of the 10 SNPs with $P < 10^{-5}$ in the combined analysis of these 114 SNPs (Table S4),

rs6129058 showed the strongest evidence of association with severe CiHFS (HR=2.40; 95%CI=1.78–3.25; $P=1.2\times 10^{-8}$) (Table 2, Table S4 and Figure S5). After adjustment for the most significant SNP rs6129058, no further variants were associated with CiHFS ($P=0.84$).

We next conducted haplotype analyses based on the four most strongly CiHFS-associated SNPs ($P<10^{-7}$; Table 2). Taking GGTT as reference, the TTAA haplotype showed similar association with risk of grade 3 CiHFS (HR=2.48, 95%CI =1.81-3.39, $P=1.43\times 10^{-8}$) suggesting that any of these 4 risk variants may be causative (Supplementary results, Table S5). Therefore, haplotype TTAA will be used to refer 4 risk alleles for the consequent analysis.

Risk haplotype is associated with reduced *CDH4* mRNA expression

To explore how the identified variants confer susceptibility to CiHFS, we first examined the genomic landscape of the risk variant-containing 20q13.33 locus. The closest gene, located ~ 90kb away, is *CDH4* and encodes R-cadherin (RCAD). Cadherin family members are master regulators of the cohesiveness of epithelial layers, and genetic defects in cadherin genes have been linked to inherited skin disorders²². Although RCAD has not been previously implicated in skin physiology, immunofluorescence revealed that RCAD is mainly localized in the suprabasal granular layer of the stratified epidermis (Figure S6). This distribution differed from that documented for other cadherin family members (Figure S6 and Figure S7) and suggested the potential involvement of *CDH4* gene expression in CiHFS. Because we were unable to assess *CDH4* mRNA expression in skin samples due to RNA degradation, we performed expression quantitative trait locus (eQTL) analyses in normal human liver tissues samples and found a significant association between the presence of the risk haplotype TTAA and reduced *CDH4* mRNA expression compared to the reference haplotype GGTT ($P=0.017$; Figure S8).

The risk locus interacts with the *CDH4* promoter

To assess the potential of each of the identified variants to affect the binding of transcription factors we used two *in silico* tools: HaploReg v4.1^{17,18} and SNP2TFBS¹⁹. Variants rs6093063, rs56152339 and rs6065060 were predicted to alter several transcription factors binding sites (TFBS) (Table S6), but the two algorithms produced different results. In addition, chromatin immunoprecipitation (ChIP)-seq data available in ENCODE²³ for normal human

epidermal keratinocytes (NHEK) showed no significant TFBS or defined enhancers overlapping with our risk variants (Figure 1A, top). However, by means of circularized chromosome conformation capture followed by high-throughput sequencing (4C-seq) we detected chromatin contacts between the risk alleles-containing region and the *CDH4* promoter in a keratinocyte cell line homozygous for the risk haplotype TTAA in two different viewpoints located in the *CDH4* gene promoter (Figure 1A, bottom and Figure S9). Importantly, we confirmed by 3C sequencing followed by Sanger sequencing that this interaction directly involved the genomic region containing the identified SNPs, in particular the rs6129058 variant, and this was significantly higher in the cell line homozygous for the risk haplotype TTAA (Figure 2). This interaction was much weaker in a keratinocyte cell line homozygous for the reference haplotype GGTT (Figure 1B, 2B, 2C and Figure S9). A third viewpoint located over the risk alleles-containing region revealed a detectable interaction that was more evident in the risk haplotype cells (Figure S10). These results suggest a regulatory mechanism by which the presence of the risk alleles causes changes in chromatin looping, which in turn can cause changes in *CDH4* gene expression. Taken together, the results from eQTL and 4C-seq analyses lead us to conclude that *CDH4* is a strong candidate to be involved in the susceptibility to CiHFS.

***CDH4*-deficiency leads to decreased levels of involucrin**

To explore the contribution of the *CDH4* gene to the proper differentiation of keratinocytes, we next turned to keratinocytes cultured *in vitro*, both under proliferative conditions and upon calcium-induced differentiation. Immunoblot analyses revealed that RCAD was not expressed under low calcium conditions, and that it was only expressed upon differentiation (Figure 3A), consistent with its primary localization at the suprabasal layer of the epidermis. We next assessed the consequences of decreasing RCAD in keratinocyte differentiation using short hairpin RNA (shRNA). While there were no changes in the expression of the differentiation markers keratin1, keratin10, filaggrin or loricrin (data not shown), we found that mRNA (Figure 3B) and protein levels (Figure 3C) of involucrin, measured 48 hours after calcium addition, were clearly reduced in the two stable RCAD knock-down (KD) clones compared to control cells.

RCAD and involucrin levels in skin are inversely correlated with risk of CiHFS

To assess the implications of these findings in the susceptibility to CiHFS, we next evaluated by immunohistochemistry the association of the risk alleles identified in our series with the expression of RCAD and involucrin in skin samples collected prior to capecitabine treatment. The presence of the risk alleles, although not reach statistically significant levels due to the sample size is not large enough to ensure adequate power, data suggest a correlation with reduced levels of involucrin and RCAD ($P_{\text{RCAD}}=0.156$, $P_{\text{involucrin}}=0.224$) (Figure S11), in agreement with *CDH4* mRNA expression levels. . Moreover, we observed an association of the expression of these proteins with the risk of suffering this adverse event, since before starting the treatment, the skin of the patients exhibiting severe toxicity (grade 3), seems to express lower RCAD ($P_{\text{RCAD}}=0.221$) and expressed significantly lower involucrin ($P_{\text{involucrin}}=0.001$) levels compared to those of patients suffering no toxicity (grade 0) (Figure 3D and 3E, respectively).

DISCUSSION

The most comprehensive analysis published to date of capecitabine toxicity pharmacogenetics revealed that polymorphisms in enzymes involved in the biochemical pathway of capecitabine biotransformation had only a modest power to predict capecitabine toxicity¹¹. Thus, the individual variability in CiHFS susceptibility remained largely unexplained and there was an urgent need to identify valuable predictive markers for patient risk stratification that could also elucidate the precise molecular mechanisms underlying this common adverse drug reaction. By combining GWAS analysis, fine-scale mapping, and functional analyses using human skin cells and tissues, we provide compelling evidence that *CDH4* regulatory variants are involved in the development of CiHFS.

The combination of GWAS and functional genomics has revealed that the vast majority of disease-associated SNPs identified by GWAS are located in non-coding genomic regions that can modulate the expression of genes located far away in the linear genome²⁴. These non-coding variants can affect gene

expression by altering the binding of transcription factors or structural proteins such as the chromatin insulator CTCF and cohesin²⁵.

Our 4C-seq experiments detected a physical interaction between the *CDH4* promoter and the risk alleles-containing region, which was significantly higher in the keratinocyte cell line homozygous for the risk haplotype. Despite the weakness of the interaction observed when we set up the viewpoint in the risk alleles-containing region, most likely due to technical limitations of the 4C technique, our 3C experiment unequivocally demonstrates the reliability of the described contact, which in addition occurs in close proximity to the most significantly associated SNP, rs6129058.

Although we do not provide direct evidence that the presence of the risk alleles is responsible for the observed changes among the contact profiles, we hypothesize that the risk alleles affect the binding of transcriptional regulators or structural proteins that positively or negatively regulate *CDH4* transcription. RCAD belongs to the cadherin family, whose members play fundamental roles in the maintenance of calcium dependent cell-cell adhesion and thus preserve proper tissue architecture and function²⁶. We found that RCAD is expressed by differentiated keratinocytes in the suprabasal layer of the epidermis; whether or not RCAD plays a role in skin physiology or pathology has not yet been investigated. Interestingly, we observed that a reduction in RCAD protein levels leads to a decrease in involucrin, a cross-linked protein in the cornified envelope - an insoluble protein structure that is essential for skin barrier function²⁷. Mutations in components of the granular layer and cornified envelope are thought to contribute to genetic susceptibility to chronic barrier defects, as in atopic dermatitis²⁸. Taken together, these findings suggest a novel involvement of RCAD in the regulation of keratinocyte differentiation through involucrin expression, although further studies are needed to explore how RCAD regulates gene expression. Involucrin levels present in the skin of the patients prior to capecitabine treatment were inversely correlated with CiHFS upon treatment, which could be a clinically useful biomarker for risk stratification of patients. We found that reduction of *CDH4* expression leads to a decrease in involucrin expression. This decrease alone may not directly alter skin function, as observed in mouse genetic models²⁹ and consistent with the lack of symptoms we observed in the skin of the patients sampled prior capecitabine

treatment. However, reductions in both RCAD and involucrin levels at the suprabasal layers may increase the cytotoxic effect of capecitabine leading to a breakdown of the skin epidermal barrier. This effect could be particularly dramatic in the palms of the hands and soles of the feet, due to the higher epidermal cell division rate, greater expression of capecitabine-metabolizing enzymes, gravitational forces and temperature gradient in the distal extremities, as well as increased drug concentration in the eccrine glands of these areas³⁰.

To date, different therapies are used for CiHFS prophylaxis (pyridoxine, regional cooling, celecoxib (Celebrex), vitamin B6, topical urea, and oral corticosteroids) and several trials have been performed to evaluate their benefit^{31–34}. However, contradictory results have been found and as yet there do not appear to be any effective therapies for the prevention of capecitabine-related HFS. Involucrin is a major protein of the cornified envelope of keratinocytes that provides much of the structural integrity of skin and its expression is stimulated by a number of agents including calcium and 1,25-dihydroxy-vitamin D3 that promote the differentiation process in keratinocytes. Taking into account the relationship observed in this study between the decreased RCAD and involucrin levels at the suprabasal layers in the skin of patients and the increased cytotoxic effect of capecitabine, we propose that the use of topical administration of calcitriol (calcitriol ointment), commonly used to treat plaque psoriasis, to palms and soles could be beneficial in preventing or decreasing the severity of this ADR, in particular in patients carrying the risk alleles.

Conclusion

In summary, using a multifaceted approach, we uncovered a novel mechanism of CiHFS susceptibility. Our results open new avenues for future research on the pathogenic mechanisms underlying CiHFS and on the clinical application of the identified risk SNPs along with the RCAD and involucrin expression as predictive biomarkers of CiHFS as a further step towards personalized cancer treatment.

Study Highlights

What is the current knowledge on the topic? To date, several factors associated with the appearance of capecitabine-induced hand-foot syndrome (CiHFS) including clinical factors (gender, age) and genetic factors (mainly variants in genes involved in the metabolic biotransformation of capecitabine) have been described. Nevertheless, they do not fully explain the whole spectrum of the interindividual variability of the risk of CiHFS appearance.

What question did this study address? The aim of this study is to identify novel CiHFS genetic risk factors.

What does this study add to our knowledge? This study uncovered a novel mechanism associated with the CiHFS susceptibility by the role of RCAD and involucrin proteins in the regulation of keratinocyte differentiation.

How might this change clinical pharmacology or translational science? Our results open new avenues for future research on the pathogenic mechanisms underlying CiHFS and on the clinical application of the identified risk SNPs along with the RCAD and involucrin expression as predictive biomarkers of CiHFS as a further step towards personalized cancer treatment.

AUTHOR CONTRIBUTIONS

S.R.P., M.N.S., R.N.T., A.C., A.L., M.P.M., R.L.M., and A.G.N. wrote the manuscript;

A.G.N., M.M. and J.B. designed the research;

M.M., J.d.T.M., L.A.L.F., N.R., J.A.G.S., E.A. D.C., L.M., S.R.P. and A.G.N performed the research;

G.P., A.C., A.K. R.N.T. A.L and A.G.N analyzed the data

G.P, M.N.S, R.L.M, S.R.P. M.L. M.P.M and S.R.P. contributed analytical tools.

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doi:10.1001/jamaoncol.2017.1269

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FIGURES

Figure 1. Chromatin interactions at 20q13.33 between *CDH4* promoter and the CiHFS susceptibility locus.

(A) Detail of the 20q13.33 CiHFS susceptibility locus located ~90kb upstream of the *CDH4* gene promoter showing, from the top down, chromosomal coordinates, a track for transcription factor binding sites (TFBS) conserved in human, mouse and rat, a track with chromatin states defined in NHEK cells from ENCODE/Broad (color code appears below), the positions of the four most strongly associated SNPs (red arrows) and the contact profile from a viewpoint (Vp2), located in the *CDH4* gene promoter (Vp, red), in cells homozygous for the risk haplotype TTAA. This profile was generated using a 30-kb window size in the main trend subpanel. High contact probability (red) is observed between the *CDH4* promoter and the region containing CiHFS-associated SNPs. (B) Using the same viewpoint in a different keratinocyte cell line homozygous for the reference haplotype GGTT, this interaction was clearly decreased. See also Supplementary Figure S8.

Figure 2. 3C validation of the identified chromatin interaction between the *CDH4* promoter and the risk alleles-containing region.

(A) Detail of 20q13.33 containing the CiHFS susceptibility locus located ~90kb upstream of the *CDH4* gene promoter. Brown arrowhead indicates the position of the top hit SNP (rs6129058). Genomic localization of the primers designed to detect the long-range chromatin interaction, two negative regions and input are represented as colored lines in the magnifications below. HindIII cutting sites in these genomic regions are shown. Electropherogram of the sequence of the 3C digestion/ligation products confirms the chromatin interaction between the *CDH4* promoter and the risk alleles-containing region. HindIII cutting site is boxed. Upstream of the cutting site the sequence corresponds to the CiHFS susceptibility locus (at 65bp distance from rs6129058); downstream, the sequence corresponds to the *CDH4* promoter-containing region. (B) Real-time PCR analysis of the chromatin interaction between the *CDH4* promoter and the risk locus for both cell lines, homozygous for the reference haplotype (GGTT) and homozygous for the risk haplotype (TTAA). Fold change was normalized to input. Two regions located upstream of rs6129058 and that show minor interaction with the *CDH4* promoter region were included as negative controls.

(C) Agarose gel electrophoresis showing the PCR products of the input and the chromatin *CDH4* promoter-risk locus interaction fragments for both cell lines, homozygous for the reference haplotype (GGTT) and homozygous for the risk haplotype (TTAA). Abbreviations: Ref: reference.

Figure 3. RCAD and involucrin protein levels in differentiated keratinocytes and in patient skin. (A) Western blot (WB) analysis of RCAD levels in HaCaT keratinocytes both in low calcium conditions (LC) and at different time points after calcium switch. (B) Real Time PCR (RT-PCR) analyses of *CDH4* (RCAD), *CDH1* (E-cadherin) and *IVN* (involucrin) mRNA levels in scramble control cells and two different RCAD knock-down (KD) clones after 48 h of calcium treatment. Data was normalized to control values and represented as mean $\pm$ SEM; ** $P < 0.005$, *** $P < 0.0001$, Student's t test ($n = 5$ independent experiments). (C) WB analyses of RCAD, E-cadherin (ECAD) and involucrin in scramble control cells and two RCAD KD clones after 48 h of calcium treatment. Boxplot of the RCAD (D) and involucrin (E) expression in the epidermis of 16 skin samples from breast cancer patients prior to capecitabine treatment suffering CiHFS grade 0 ($n=4$) or CiHFS grade 3 ($n=12$). Skin samples were taken before capecitabine treatment. Differences in RCAD and involucrin expression were assessed using Student's t test ($P_{\text{RCAD}} = 0.221$; * $P_{\text{involucrin}} = 0.001$).

Supplementary Information Titles

1 - Supplementary information File

Table 1. Summary of Patient Characteristics

Clinical Characteristics	Discovery cohort (n=166)			Replication cohort (n=85)		
	Control (n=78)	Cases (n=88)	P value	Control (n=61)	Cases (n=24)	P value
Age (yr.) at diagnosis, median (range)	70 (29 – 83)	64 (31 – 88)	0.0187	66 (36 – 87)	65 (49 – 77)	0.5222
Gender, No. (%)			0.0015			0.0064
Female	60 (76.9)	82 (93.2) ^a		30 (49.2)	20 (83.3)	
Male	18 (23.1)	5 (5.7) ^a		31 (50.8)	4 (16.7)	
Cumulative capecitabine dose, median (range), x10 ³ mg/m ²	260 (33-1775)	117 (15-455)	1.008x10 ⁻⁸	301 (35-1767)	161 (34-543)	0.0056
Capecitabine treatment ^b , No (%)			0.01524			0.0001
Standard	74 (94.9)	72 (81.8)		58 (95.1)	14 (58.3)	
Continuous	4 (5.1)	16 (18.2)		3 (4.9)	10 (41.7)	
Primary site, No (%)			0.0052			1.18x10 ^{-8d}
Breast	48 (61.5)	71 (80.7) ^a		8 (13.1)	19 (79.2)	
Colorectal	30 (38.5)	16 (18.2) ^a		53 (86.9)	5 (20.8)	
Patient recruitment, No (%)			0.370			1.18x10 ^{-8d}
H.U. San Carlos (Madrid)	38 (48.7)	49 (55.7)		-	-	
H. U. Virgen de la Victoria (Málaga)	40 (51.3)	39 (44.3)		-	-	
H.U. Gregorio Marañón (Madrid)	-	-		53 (86.9)	5 (20.8)	
Clinical Trial ^c	-	-		8 (13.1)	19 (79.2)	

^a One individual with unknown data. ^bStandard regimen: 1250 mg/m² orally every 12 h on days 1-14, every 3 weeks. Continuous regimen: 800 mg/m² orally every 12 h daily during 21 days. ^cClinicalTrials.gov Identifier: NCT00418028; <https://clinicaltrials.gov/>. ^dData for primary site and patient recruitment showed same distribution.

Table 2. Association results for the two stages and the combined analysis at the 20q13.33 locus for the 3 most strongly associated variants and the original GWAS hit rs6093063

Variant	Position *	Cohort	MAF	P	HR	95%CI
rs6093063	20:59723157	Discovery	0.17	5.9×10^{-6}	2.17	1.55-3.04
		Replication	0.18	0.011	2.27	1.20-4.30
		Combined	0.17	1.3×10^{-7}	2.17	1.63-2.88
rs6129058	20:59732322	Discovery	0.18	1.3×10^{-6}	2.46	1.71-3.54
		Replication	0.21	2.9×10^{-3}	2.70	1.40-5.19
		Combined	0.19	1.2×10^{-8}	2.40	1.78-3.25
rs56152339	20:59732787	Discovery	0.18	4.8×10^{-6}	2.35	1.63-3.39
		Replication	0.19	6.8×10^{-3}	2.45	1.28-4.69
		Combined	0.19	7.4×10^{-8}	2.30	1.70-3.12
rs6065060	20:59735681	Discovery	0.18	4.8×10^{-6}	2.35	1.63-3.40
		Replication	0.20	6.9×10^{-3}	2.49	1.28-4.69
		Combined	0.19	7.6×10^{-8}	2.30	1.70-3.12

Associations between SNPs and CiHFS were assessed by Cox regression analyses adjusted for sex, tumor type and treatment regimen. An additive model of inheritance was assumed. HRs are per copy of the minor allele. * Chromosome positions are based on Genome Reference Consortium Human Build 37 (GRCh37/hg19). Abbreviations: MAF, minor allele frequency; HR, hazard ratio; CI, confidence interval.

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Title:

Regulatory CDH4 Genetic Variants Associate With Risk to Develop Capecitabine-Induced Hand-Foot Syndrome.

Date:

2021-02

Citation:

Ruiz-Pinto, S., Pita, G., Martín, M., Nuñez-Torres, R., Cuadrado, A., Shahbazi, M. N., Caronia, D., Kojic, A., Moreno, L. T., de la Torre-Montero, J. C., Lozano, M., López-Fernández, L. A., Ribelles, N., García-Saenz, J. A., Alba, E., Milne, R. L., Losada, A., Pérez-Moreno, M., Benítez, J. & González-Neira, A. (2021). Regulatory CDH4 Genetic Variants Associate With Risk to Develop Capecitabine-Induced Hand-Foot Syndrome.. Clin Pharmacol Ther, 109 (2), pp.462-470. <https://doi.org/10.1002/cpt.2013>.

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