Cancer risk in DM1 is sex-related and linked to miRNA-200/141 downregulation

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ABSTRACT

Objective: Describe the incidence of cancer in a large cohort of patients with myotonic dystrophy type 1 (DM1) and to unravel the underlying molecular mechanisms.

Methods: Standardized incidence ratios (SIRs) were calculated in the Gipuzkoa DM1 cohort (1985–2013), dividing observed numbers by expected numbers for all cancers combined and stratified by sex. An estimation of the expected incidence was achieved by multiplying the age- and sex-specific incidence rates from the Basque population cancer registry by the person-years observed in the study cohort. Large-scale gene expression of peripheral blood mononuclear cell samples derived from 10 individuals with DM1 (5 men, 5 women) and 10 healthy matched controls was analyzed by the Human Gene 1.0 ST Affymetrix microarray.

Results: During 18,796 person-years of follow-up, corresponding to 424 patients with DM1, we observed 70 cancers in 62 patients giving a 1.81-fold risk (95% confidence interval [CI] 1.37–2.36), which was stronger in women than in men. Ovary (SIR 8.33, 95% CI 1.72–24.31) and endometrium (SIR 6.86, 95% CI 2.23–16.02) in women and thyroid (SIR 23.33, 95% CI 9.38–48.08) and brain (SIR 9.80, 95% CI 3.18–22.88) in both sexes were tumor sites with significantly higher risks in DM1. There were differences in gene expression between healthy controls and patients with DM1 and between men and women with DM1; all patients with DM1 combined and female patients with DM1 displayed significant downregulation of the microRNA (miRNA)-200c/141 tumor suppressor family.

Conclusions: Oncologic risk is increased in DM1, especially in women and for gynecologic, brain, and thyroid cancer. Expression of the miRNA-200/miRNA-141 tumor suppressor family is decreased in women with DM1. *Neurology*® 2016;87:1250–1257

GLOSSARY

CI = confidence interval; **DM1** = myotonic dystrophy type 1; **DM2** = myotonic dystrophy type 2; **ICD** = *International Classification of Diseases*; **miRNA** = microRNA; **MIRS** = Muscular Impairment Rating Scale; **SIR** = standardized incidence ratio.

Myotonic muscular dystrophies are a group of autosomal dominant, multisystem diseases encompassed by 2 subtypes. Myotonic dystrophy type 1 (DM1), also known as Curschmann-Steinert disease (OMIM: #160900), is caused by the expansion of an unstable trinucleotide (CTG) repeat expansion in the 3′ untranslated region of a *DMPK* kinase gene located in chromosome 19. The type 2 (DM2) (OMIM #602668) is originated by a tetranucleotide (CCTG) repeat expansion in intron 1 of the *CNBP* gene. DM1 displays a more severe phenotype than DM2 and represents the most common adult muscular dystrophy, with an estimated prevalence ranging from 0.5 to 18 of 100,000 people,[1] although the disease prevalence is higher in some regions such the province of Gipuzkoa in the Spanish Basque Country.[2]

The DM1 phenotype is grossly related to the size of the CTG expansion and shows an extremely wide variability ranging from very mild forms with CTG repeats less than 50 and

Supplemental data at Neurology.org

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usually associated with cataracts developed in presenile age, to severe neonatal forms with more than CTG 1,000 repeats, associated with severe developmental delays. Life expectancy is reduced because of complications derived from muscle weakness, respiratory and cardiac involvement, neoplasms, and metabolic disturbances such as hypercholesterolemia or diabetes.[3,4]

In 1965, Cantwell and Reed[5] first reported an association between DM1 and pilomatricoma, a rare and benign cutaneous tumor. Since then, several case reports describing benign and malignant neoplasms in virtually any location have been published.[6] Furthermore, in recent years, large epidemiologic studies performed in population- or clinical-based cohorts provided evidence of increased risk of malignant tumors in patients with DM.[7–10] Excess risks of endometrium, ovarian, thyroid, skin, eye, and colon cancer were observed in 2 of the 3 studies, while brain cancer excess risk was observed in one study. A follow-up study including 911 patients with DM showed that females with DM1 were more likely to develop cancers.[11] Several studies have shown no association between the size of leukocyte repeat expansion and cancer risk in those patients[6] and the underlying causes, and the biological mechanisms of the susceptibility for developing tumors are still unknown.

In this study, we quantified cancer risk in the clinically and genetically well-characterized Gipuzkoa Myotonic Dystrophy Cohort, and used gene expression analysis to identify possible molecular mechanism of cancer susceptibility in those patients. The cohort includes all patients diagnosed with DM in the past 30 years in an area with one of the largest DM1 prevalences worldwide.

METHODS

Study population. Data from patients were retrospectively obtained from the medical records of the Gipuzkoa historical myotonic dystrophy cohort, established in 1985. We identified 503 patients with a molecularly confirmed DM diagnosis between 1985 and 2013. We excluded 4 patients with DM2 diagnosis and 75 patients with DM1 because of lack of data or they were lost to follow-up.

From patients' medical records, we extracted the following information: sex, age at the time of study enrollment and age at death, nucleotide expansion size (CTG triplets), calendar year of diagnosis, disease severity assessed using the Muscular Impairment Rating Scale (MIRS)[12] at the time of the last visit, age and calendar year of cancer diagnosis, and cancer anatomical site. Diagnoses of all types of malignant neoplasia were coded using ICD, Ninth Revision. Death causes were coded using ICD-10 (2014 version).[13]

Genetic analysis. The CTG repeat was measured at the time of DM1 diagnosis by conventional PCR and Southern blot. Conventional PCR was performed with 100 ng of genomic DNA using gene-specific primers flanking the *DMPK* CTG repeat. All normal homozygotes and expanded alleles were confirmed with Southern blot. We included patients with 40 or more CTG repeats because of the demonstrated instability of the fragment size from this threshold, independently of the presence or absence of clinical manifestations, assuming that if we eliminate the cases considered as premutations, we neglect a possible effect of genomic condition over cancer prone.

Statistical analysis. Follow-up started at the date of DM1 diagnosis and ended at the date of first cancer diagnosis, death, or last visit. We calculated standardized incidence ratios (SIRs) by dividing the observed numbers of cancer by the expected numbers for all cancers combined and cancer-specific anatomical sites, overall and stratified by sex. Expected numbers were calculated by multiplying the age- and sex-specific incidence rate from the Basque population cancer registry[14] by the person-years of the study cohort. SIRs were obtained for all types of cancers except for basal cell carcinoma of the skin because this type of cancer is not collected in the registry. Confidence intervals (CIs) were calculated using the Poisson distribution.

Mean repeat length was compared for patients with and without cancer using the Student t test. All tests were considered statistically significant if $p < 0.05$. Statistical analysis was performed using Stata/SE 12 (StataCorp LP, College Station, TX).

Transcriptomic analysis. This analysis included 10 patients with DM1 (5 women, 5 men; mean age: 43.4 ± 5.16 years; expansion size: 750 ± 306 CTG triplets) who had moderate or severe clinical manifestations (MIRS median score = 3, range 3–5) and no malignancy before or at the time of blood collection and 10 age- and sex-matched healthy controls. We extracted RNA from peripheral blood mononuclear cells using the LeukoLOCK Total RNA Isolation System (Life Technologies, Carlsbad, CA). For the RNA extraction, we use a 2-step protocol, first with the miRNesay Mini Kit (QIAGEN, Valencia, CA) followed by automated RNA extraction in the QIAcube. Large-scale gene expression was measured by the Human Gene 1.0 ST Affymetrix microarray (Affymetrix, Santa Clara, CA). RNA integrity was checked with an Agilent RNA 6000 Nano Kit (Agilent Technologies, Inc., Santa Clara, CA). Samples with an RNA integrity value above 7 were accepted to be processed. Three hundred nanograms of total RNA were used for microarray analysis following the manufacturer's instructions.

We analyzed gene expression differences in patients with DM1 and healthy controls, and in a second step, we studied the differences between patients with DM1 by sex. Results of the microarray data were extended by reverse transcription–PCR in an additional DM1 male (n = 16) and female (n = 25) subset of patients.

Western blot analysis. We did a Western blot of the protein products of the targets altered in the transcriptomic analysis. Immunoblots were performed as previously reported.[15] We used ab16123 (Abcam, Cambridge, UK) for $p16^{Ink4a}$ detection and A-5441 (Sigma, St. Louis, MO) for β-actin and horseradish peroxidase–linked anti-rabbit or anti-mouse (Santa Cruz Biotechnology, Dallas, TX) secondary antibody at a 1:2,000 dilution. Detection was accomplished by chemiluminescence using Novex ECL Chemi Substrate (ThermoFisher Scientific, Waltham, MA).

Standard protocol approvals, registrations, and patient consents. This study was approved by the Donostia Universitary

Hospital Ethical Board and conducted in accordance with the Declaration of Helsinki ethical standards.

RESULTS **Cancer incidence rate in patients with DM1.** The study included 424 patients with DM1; 214 (50.5%) of them were women. Mean CTG repeat expansion size at the time of DM1 diagnosis was 684 ± 535 CTG triplet expansion (range: 43–2,000 CTG repeats). One hundred thirty-seven patients were deceased at the time of analysis. Additional demographic and clinical features of the patients are shown in table 1. The most common causes of death were diseases of the respiratory system (38%), the circulatory system (24.1%), and neoplasms (15.3%); causes of death were unknown in 15.3% of the patients (table e-1 at Neurology.org).

During 18,796 person-years of follow-up, we observed 70 cancers in 62 patients with DM1 (32 women, 30 men; table e-2). We observed multiple primary cancers in 7 patients (6 patients had 2 cancers and 1 patient had 3 different cancers) (table e-3). Mean age at cancer diagnosis for all 62 patients was 46.6 ± 14.7 years. Mean CTG repeat expansion size in patients with DM1 and cancer was 629 ± 79 CTG repeats and there were no statistical differences between the length of the CTG expansion in DM1 patients with or without cancer ($p > 0.05$). Although we only had MIRS data available at the time of the study in 217 patients, there were no statistical differences regarding cancer status ($p > 0.05$). Digestive organs (24.8%), genitourinary system (21.4%), skin (12.8%), and thyroid gland (11.4%) were the most frequent sites for malignant tumors (table e-2).

When compared to the general population from Gipuzkoa, we detected an approximately 2-fold increase in risk of cancer (SIR 1.81, 95% CI 1.37–2.36). This risk increase was stronger in women (SIR 2.71, 95% CI 1.77–3.97) than in men (SIR 1.40, 95% CI 0.94–2.01). When we compared the anatomical site of the cancer in the DM1 population with the general population stratified by sex and age, we found a high risk of developing ovary (SIR 8.33, 95% CI 1.72–24.31) and endometrium (SIR 6.86, 95% CI 2.23–16.02) cancer in women and thyroid (SIR 23.33, 95% CI 9.38–48.08) and brain (SIR 9.80, 95% CI 3.18–22.88) cancer in both sexes (table 2). Besides these malignant tumors, only colorectal cancer nearly reached statistical significance (SIR 2.06, 95% CI 0.94–3.92).

Table 1 Clinical and molecular characteristics of the myotonic dystrophy type 1 cohort

No. of patients	424
Sex	214 women (50.5%), 210 men (49.5%)
Mean CTG repeat expansion size	684 ± 535.6 CTG repeats (43–2,000 CTG repeats)
Transmission	Paternal 65%
MIRS score (n = 217)	2.54 ± 1.21
Age at last visit, y	50.7 ± 15.61
Respiratory symptoms	86 patients (20.3%)
Cardiac disease	184 patients (43.4%)
Patients with cancer	62 (14.6%)
Death	137 patients
Mean age at death, y	60.25 ± 13.11
Follow-up days	18,313.6 ± 5,739.2

Abbreviation: MIRS = Muscular Impairment Rating Scale.

Transcriptomic analysis of patients with DM1. As previously described,[16] we observed differences in gene expression between healthy men and women (figure 1, A and B). Similar sex differences were detected in the transcriptome of patients with DM1 (figure 1, A–C). Compared to healthy controls, patients with DM1 had an upregulation of RNA5SP211, an unknown pseudogene, and downregulation of TAS2R13, a subtype of taste receptors.

When we analyzed the results according to sex, we found that EMR4P (a hormone receptor), CD24 (a glycoprotein expressed on mature granulocytes and B cells), PLA2G7 (a platelet activating factor), and caspase-5 (a gene implicated in apoptosis) were significantly upregulated in men (figure 1, B and C). Moreover, we identified 11 genes differentially expressed in women with DM1, including upregulation of a subtype of histone (HIST1H2AK) and pyruvate dehydrogenase kinase 4 (PDK4), and downregulation of 3 microRNA precursors (pre-miR-3978, pre-miR-141, and pre-miR-200c), a transcription factor (ZEB2), an olfactory receptor (OR52K2), an inhibitory receptor of myeloid cells (CLEC12B), myoferlin (a protein that has a role in calcium-mediated membrane fusion events, membrane regeneration and repair, and subsequently in muscle weakness), a death-associated protein kinase (DAPK1), and MS4A4E (figure 1, B and C). The same results were observed in an independent validation set of patients (figure 1, D and E). Specifically, the differentially expressed levels of 13 of 17 genes detected in the array were confirmed in the validation cohort.

Identification of *miR-200c/miR-141* cluster associated with the cancer susceptibility phenotype in DM1. The miR-200c/miR-144 and miR-3978 precursors were downregulated in the group of women with DM1 in the array and validated in the extended cohort (figure 2, A and B). Of note, the mature forms miR-200c-5p, miR-141-3p, miR-141-5p, and miR-3978 were downregulated in the cohort of women with DM1 (figure 2C). On the contrary, the expression was slightly elevated in men, revealing a sex differential expression of those miRs in DM1 (figure 2, C and D).

Table 2 SIRs by anatomical site

Cancer	Age at diagnosis of cancer, y	O	E	SIR	95% CI
Women					
Ovary	48 (16-63)	3	0.36	8.33	1.72-24.31[a]
Endometrium	53 (38-59)	5	0.73	6.86	2.23-16.02[a]
Breast	57 (56-69)	3	2.88	1.04	0.21-3.04
Men					
Prostate	75 (73-77)	2	4.33	0.46	0.06-1.67
Testes	15	1	0.07	14.25	0.35-79.6
Both sexes					
Thyroid	51 (40-67)	7	0.30	23.33	9.38-48.08[a]
Brain	53 (30-56)	5	0.51	9.80	3.18-22.88[a]
Kidney	53.5 (40-67)	2	0.79	2.53	0.31-9.15
Non-Hodgkin lymphoma	65.5 (61-70)	2	0.70	2.86	0.35-10.32
Liver	69 (66-72)	2	0.79	2.54	0.31-9.15
Colorectum	59 (41-86)	9	4.36	2.06	0.94-3.92
Stomach	56.5 (55-58)	2	1.27	1.57	0.19-5.69
Lung	59.5 (47-73)	4	3.40	1.18	0.32-3.01
Leukemia	57	1	0.26	3.80	0.09-21.43
Head and neck	43	1	1.07	0.94	0.02-5.21
Melanoma	34	1	0.58	1.72	0.04-9.61
Urinary bladder	64	1	1.56	0.64	0.02-3.57

Abbreviations: CI = confidence interval; E = expected cancer in the Basque general population adjusted for sex and age; O = observed cancer in myotonic dystrophy type 1 cohort; SIR = standardized incidence ratio.
[a] Indicates statistical significance, $p < 0.05$.

Because the *BMI1* and *ZEB* family member oncogenes are reported as miR-200 target genes,[17–22] we measured their expression finding that ZEB1 and ZEB2 were decreased while BMI1 levels were upregulated in the group of women with DM1 (figure 2E). Moreover, the expression of $p16^{Ink4a}$ tumor suppressor, a known target of BMI1 epigenetic silencing, was lower at both messenger RNA and protein level in the same group of women with DM1 (figure 2, F and G).

DISCUSSION There is recent accumulating evidence that patients with DM1 are at high risk of developing cancers. Confirming these findings in an independent study and in different DM populations is important for patient clinical management. Using data from a large molecularly confirmed cohort of patients with DM1, we showed a statistically significant excess risk of endometrium, ovary, thyroid, and brain cancer. In the largest epidemiologic cohort published so far accounting for more than 1,600 patients with DM1 and DM2, Gadalla et al.[7] found an apparent high risk of cancers of the endometrium, brain, ovary, and colon, and possibly thyroid and choroidal melanoma and nonmelanoma skin cancers. In smaller studies, Win et al.[8] reported a higher risk of developing thyroid cancer and choroidal melanoma whereas Mohamed et al.[9] concluded that there was a high risk of thymoma, gynecologic, and lung cancer. Recently, in a small published cohort, Bianchi et al.[10] found that skin, thyroid, ovary, and breast cancers were most frequent in patients with DM.

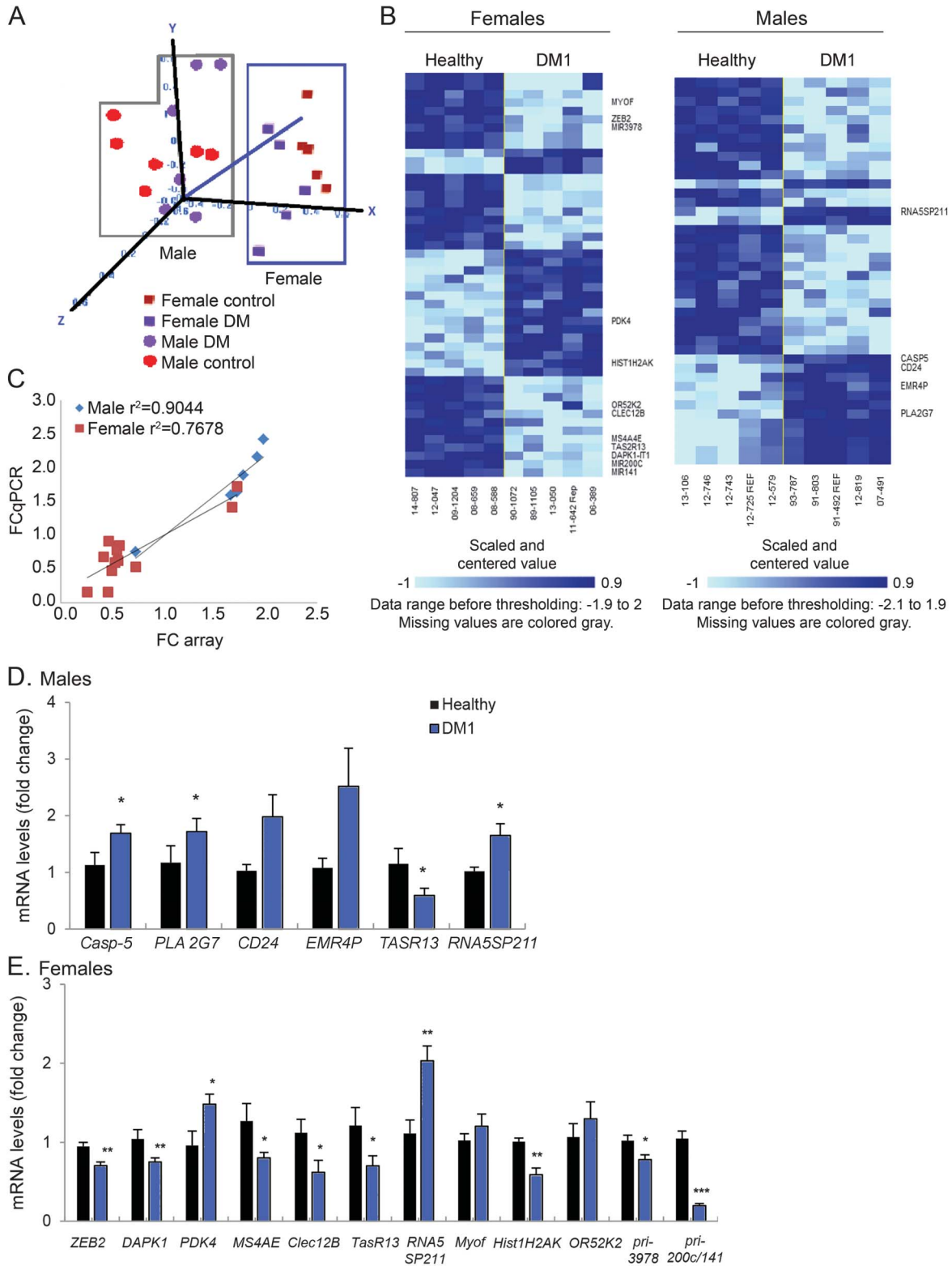
Our results are coincidental with those reported by Gadalla et al. in a population-based study of more than 1,600 patients with DM[7] with the exception of colorectal cancer. However, the CI (0.94–3.92) of this type of cancer was near to significance values and the smaller sample size in our cohort could explain this result. Of note, colorectal cancer was also the most frequent among those patients who had 2 or more cancers. The excess risks of thyroid,[8,10] endometrium, and ovary[9,10] were also found in smaller studies. Of importance, and as also indicated by Gadalla et al.,[4,7] we did not find an overrepresentation of screening-related cancers (i.e., breast and prostate), strongly suggesting that the results are genuine and not biased by the close medical surveillance in patients with DM. The fact that there are no incidence and mortality cancer differences between the Basque and other regions in Spain[23] led us to eliminate the notion that the excess cancer in this population could be explained by other genetic factors rather than the DM1 condition itself.

Our data show that malignancies developed at a mean age of 46.6 ± 14.7 years. In agreement with previous reports,[3,24] cancers represented the third leading cause of death after respiratory and circulatory diseases in our DM1 population. A progressive increase in cumulative incidence of cancer mortality has been previously reported (2% by age 50 to 6% by age 70).[4]

Another striking feature is the apparent absence of a correlation between cancer risk and nucleotide repeat length. This result could be potentially biased for methodologic reasons because the CTG repeat expansion size was measured years before cancer emergence and also because of the somatic instability that characterizes DM1. Based on the differences found between tumoral and healthy tissues in patients with DM1, some authors have suggested that there might be an underlying independent mechanism of somatic instability in the tumoral tissue.[25–27] However, the later contrast with the recent finding that MBLN1, a splicing factor sequestered in the nuclear foci of DM1 cells, which correlates with CTG expansion size, could be implicated in the physiopathology of the progression of certain cancers.[28]

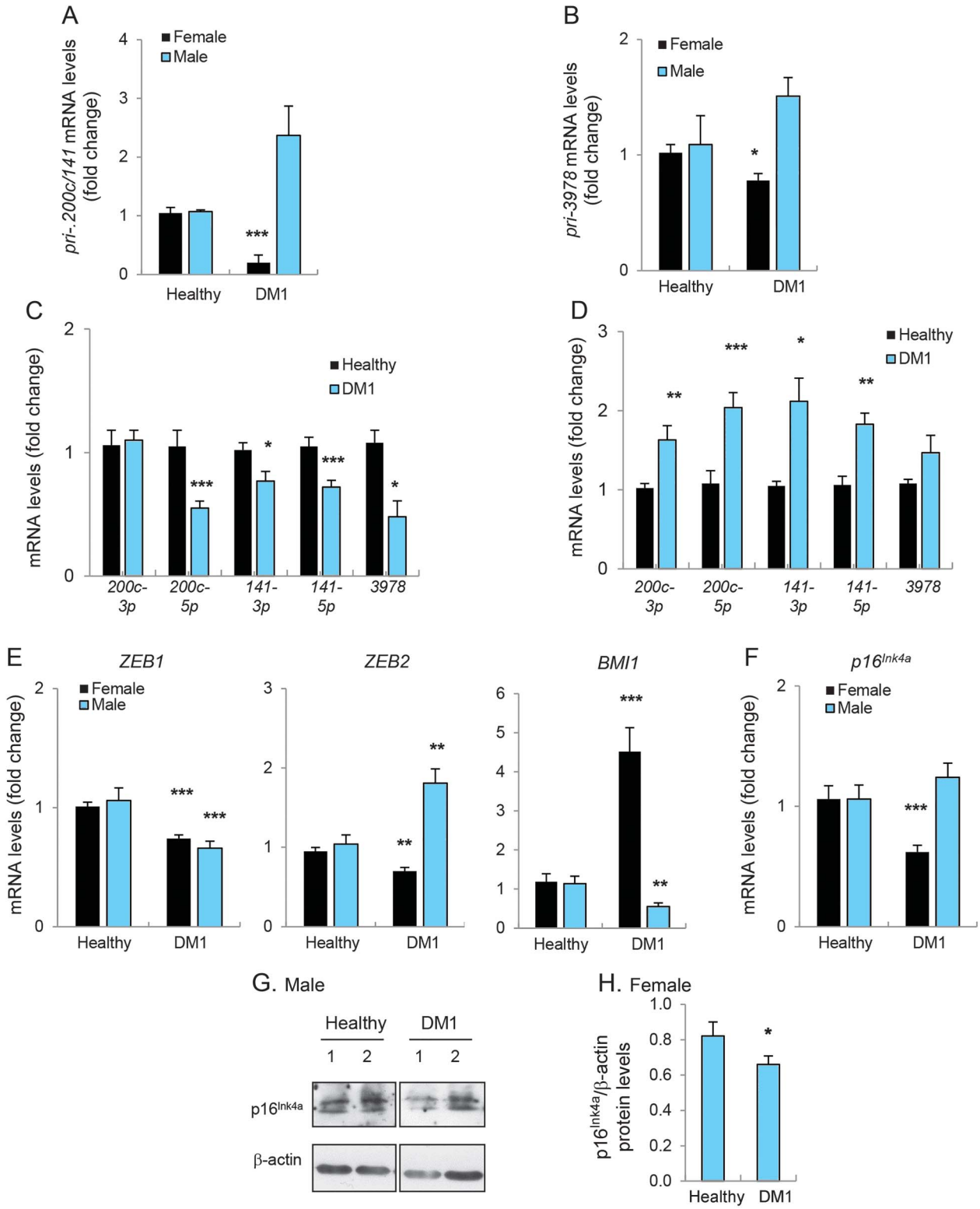
Our results showing a differential transcriptional profiling of several genes previously linked to cancer, such as *PDK4*,[29] *DAPK1*,[30] *CASP5*,[31] and *PLA2G7*[32] in patients with DM1 compared to healthy controls, open the door to a mechanistic explanation of this increased oncogenic risk in DM1. In addition, we observed a downregulation of miR-200c, miR-141, and miR-3978 mature and

Figure 1 Sex-dependent mRNA expression pattern in patients with DM1



(A) Dendrogram explaining the sex differences between the expression patterns. (B) Heatmap with the genes differentially expressed between patients with DM1 (n = 10) and healthy controls (n = 10) in women (left, n = 5) and men (right, n = 5). (C) Chart of the validation data (fold change from arrays vs fold change from reverse transcription–quantitative PCR) in women (red) and men (blue). (D) mRNA levels of genes altered in the male cohort (n = 16) compared with healthy controls (n = 5). (E) mRNA levels of genes altered in the female cohort (n = 25) compared with healthy controls (n = 5). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. DM1 = myotonic dystrophy type 1; mRNA = messenger RNA.

Figure 2 miR-200c/141 cluster and its targets in patients with DM1



(A, B) mRNA expression of 2 miRNA precursors, *miR-200c/141* and *miR-3978*, which are downregulated in women with DM1 (n = 25) and upregulated in men with DM1 (n = 16). (C, D) Mature forms of these miRNAs are downregulated in women with DM1 (left, n = 25) and upregulated in men with DM1 (right, n = 16). (E) In these groups of patients, *ZEB1* is downregulated in both sexes, while *ZEB2* is downregulated only in women with DM1. On the contrary, *BMI1* is upregulated in women and downregulated in men with DM1. (F) *p16^{Ink4a}* expression is decreased in women with DM1 (n = 25). (G) Representative immunoblots and quantification of p16^{Ink4a} derived from 2 different blood samples of female healthy controls and DM1 patients (n = 4). (H) p16^{Ink4a} protein quantification in the blots shown in G. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. DM1 = myotonic dystrophy type 1; miRNA = microRNA; mRNA = messenger RNA.

precursor forms in female patients with DM1. Of note, 2 of them, miR-200c and miR-141, belong to the same miR-200 tumor suppressor cluster. Lower levels of the miR-200c family members were detected in tumor tissues and blood-derived samples in a wide range of cancer types.[33] This decline was associated with tumor progression and poor prognosis including metastasis.[34,35]

The miR-200 family has several well-described oncogenes as downstream targets, likely the *ZEB* family and the polycomb group gene *BMI1* becoming crucial targets.[18–21,36] Our results show that these genes are altered in both sexes of patients with DM1 supporting the relevance of this molecular signaling on the disease. In particular, women with DM1 express elevated *BMI1* levels, while *ZEB1* and 2 are decreased. The existence of such alterations in patients with severe disease and no cancer prompted us to hypothesize that the downregulation of miR-200 cluster members have a role in cancer susceptibility, and the consequent upregulation of *BM1* instead of through *ZEB* may be required for its development. In support of this idea, (1) ZEB factors are epithelial–mesenchymal transition inducers and their role has been associated with metastasis rather than tumor formation; (2) the expression of $p16^{Ink4a}$ tumor suppressor, a critical target of BMI1, inversely correlates with BMI1 levels in patients with DM1; and (3) miR-200 and BMI1 play an important role in the maintenance of adult stem cells and tumor-initiating cells in many organs. Of note, many of the cancers detected in patients with DM1 emerge in tissues with a high rate of cellular replications.

Taken together, our findings support a model through a coordinated action between the miR-200 expression and its downstream targets ZEB1/2 and BMI1. In support of this notion, there is a close functional link between the miR-200 family and ZEB factors and BMI1 in a double-negative feedback loop, respectively. Thus, the activation of one of them affects the expression and activity of the others.[37,38]

Our study strength includes the population-based design (all patients with DM1 in the Gipuzkoa population were included), and therefore no selection bias exists. We are limited by the lack of information of known cancer risk factors such as smoking, diet, lifestyle, environmental influences, and alcohol intake among others. Patients with DM1 have more obesity and the prevalence of tobacco smoking is higher than in the general population,[39] so these risk factors might have had a confounding role in our results and should be considered when planning longitudinal studies. However, a recent study suggested that lifestyle factors in patients with DM1 do not explain the observed excess risk of cancers.[10]

We believe that our study provides independent cancer site–specific confirmation of recently reported excess cancer risks as part of the DM1 phenotype, especially for women, and suggests that this association could be more related to a transcriptomic regulation of oncogenic pathways than a direct consequence of the DM genomic signature.

AUTHOR CONTRIBUTIONS

Roberto Fernández-Torrón: had the original idea, collected clinical data from the patients, evaluated the patients, did the clinical and statistical analysis, and wrote the manuscript. Mikel García-Puga: performed the transcriptomic analysis and wrote the manuscript. José-Ignacio Emparanza: performed the statistical analysis, reviewed the ethics, and wrote the manuscript. Miren Maneiro: collected clinical data, evaluated the patients, and revised the manuscript. Ana-María Cobo: performed the molecular analysis and revised the manuscript. Miren Zulaika: collected clinical and molecular data, performed the transcriptomic analysis, and revised the manuscript. Juan-José Poza: collected clinical data, evaluated the patients, and revised the manuscript. Juan-Bautista Espinal: collected clinical data, evaluated the patients, and revised the manuscript. Irune Ruiz: performed the anatomopathologic analysis and revised the manuscript. Loreto Martorell: performed the molecular analysis and revised the manuscript. David Otaegui: performed the transcriptomic analysis and revised the manuscript. Ander Matheu: had the original idea, performed the transcriptomic analysis, and wrote the manuscript. Adolfo López de Munain: had the original idea, collected clinical data from the patients, followed and evaluated the patients, performed the clinical analysis, and wrote the manuscript.

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DISCLOSURE

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