

Increased incidence of endometrioid tumors caused by aberrations in E-cadherin promoter of mismatch repair-deficient mice

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Loss of E-cadherin expression is a critical step in the development and progression of gynecological tumors. Study of the precise role of E-cadherin has been hampered by the lack of satisfactory mouse model for E-cadherin deficiency. Likewise, DNA mismatch repair (MMR) is implicated in gynecological tumorigenesis, but knockout of MMR in mice predominantly causes hematologic neoplasms. Here, we show that combined disruption of E-cadherin and DNA MMR pathways increases incidence of endometrioid tumors in mice. Twenty percent of mice knockout for Msh2 enzyme and hemizygous for E-cadherin [*Msh2*(-/-)/*Cdh1*(+/-)] developed endometrioid-like tumors in the ovary, uterus and genital area. Characteristic of these tumors was a complete loss of E-cadherin expression. Sequence analysis of E-cadherin promoter region demonstrated that the loss of E-cadherin expression is caused by inactivating mutations, implying that E-cadherin is a mutational target in Msh2-deficient mice. In addition, *Msh2*(-/-)/*Cdh1*(+/-) mice showed a reduction in overall survival as compared with their *Msh2*(-/-) counterparts due to the development of more aggressive lymphomas, suggesting a specific role of E-cadherin in lymphomagenesis. In conclusion, *Msh2*(-/-)/*Cdh1*(+/-) mice provide a good model of gynecological tumorigenesis and may be useful for testing molecular target-specific therapies.

Introduction

Both deficiency in DNA mismatch repair (MMR) and aberrations in E-cadherin- β -catenin pathways have been independently implicated in tumorigenesis of solid tumors. MMR in mammalian cells serves as a major safeguard for genome integrity and cancer prevention. Its main function is to recognize and correct postreplicative errors (1,2). Consistent with the mutator phenotype hypothesis for cancer development (3), defects in MMR are known to lead to mutations at other sites genomewide (1) that, in turn, are thought to initiate malignant transformation. Two major complexes Msh2/Msh6 and Msh2/Msh3 mediate recognition of mismatch entities. The former is responsible for repair initiation of simple base mismatches, single base insertions and deletions, whereas the latter recognizes small loops. Following recognition by Msh2/Msh3 or Msh2/Msh6, the accessory heterodimers composed of either Mlh1/Pms2 or Mlh1/Mlh3 are recruited to DNA mismatch site to coordinate downstream steps (1). Loss of functional MMR results in 50- to 1000-fold increase in mutation rate throughout genome (3). Defects in MMR are associated with 70–80% of hereditary non-polyposis colorectal cancer (HNPCC) and 15% of sporadic cases of colon cancer (4–6). The HNPCC patients also have an increased risk of other carcinomas, including endometrial, ovarian, hepatobiliary and genitourinary (6). Women with

Abbreviations: EC, endometrial cancer; MMR, mismatch repair; OC, ovarian cancer; OSE, ovarian surface epithelium.

HNPCC, i.e. defects in MMR, have 10-fold, the normal risk of ovarian cancer (OC) (6) and risk of endometrial cancer (EC) equal or greater than the risk of colon cancer (7).

Inactivation of one of MMR proteins in mouse models leads mostly to the development of hematologic tumors (8). Only homozygous mutants were prone to cancer, whereas heterozygous mice remained healthy and did not develop tumors. A knockout mouse lacking Msh2 showed the most severe phenotype (8). These animals developed tumors, mostly T-cell lymphomas, at an early age, between 2 and 5 months. A small fraction of *Msh2*(-/-) mice showed tumors in gastrointestinal tract, skin and occasionally, at some other sites later in life (8). Tumors of all *Msh2*(-/-) mice displayed microsatellite instability (8) similar to human cancer cases. The consequences of MMR deficiency in cell lines generated from *Msh2*(-/-) mice included loss of heterology-dependent recombination and tolerance to methylating agents (8).

E-cadherin is a transmembrane glycoprotein, critical in maintaining the organized structure of normal epithelial cells by providing polarity and by forming tight junctions, which limit cell proliferation and migration (9). The cytoplasmic portion of E-cadherin binds catenin proteins. In normal cells binding of β -catenin to E-cadherin and APC complex inhibits signaling through the canonical Wnt pathway. E-cadherin bound to β -catenin competes with β -catenin-activated Tcf/Lef-1 pathway (9). As a transcriptional coregulator, β -catenin together with proteins of the TCF/Lef-1 family controls expression of genes implicated in cell proliferation and transformation (9–11). E-cadherin is often downregulated or lost during cancer progression (12,13). Multiple mechanisms are responsible for repression of E-cadherin function, including mutations, promoter methylation, transcription inhibition by repressors, epigenetic silencing by the polycomb complex and degradation (14–16). Loss of E-cadherin is a key event in epithelial–mesenchymal transition (17). The consequences of E-cadherin loss have been reported to include promotion of tumor invasion and metastases (18).

The significance of E-cadherin status in gynecological tumors is well established (17,19,20). E-cadherin is inactivated in 80–90% of type II EC, which are characterized by a progressive behavior and deep myometrial invasion (19). Normal ovarian surface epithelium (OSE), one of the sites where OC is thought to arise, expresses little if any E-cadherin (21). OSE cells are cells with a complex phenotype; they express both mesenchymal and epithelial markers (20,21). Depending on environmental conditions, OSE can differentiate along different pathways, suggesting that even at its mature state OSE remains somewhat pluripotent and undifferentiated (17). The level of E-cadherin in OSE rises during initial stages of neoplastic transformation indicating transition to more epithelial phenotype first (20). The reacquisition of mesenchymal features including the loss of E-cadherin in ovarian tumor cells occurs in late metastatic stage (17,21).

There is no mouse model to recapitulate tumorigenesis as it happens in humans with respect to changes associated with epithelial–mesenchymal transition including those that related to expression of mesenchymal and epithelial cadherins. This is because E-cadherin knockout animals die *in utero* on one hand, and tissue-specific loss of E-cadherin alone, on the other hand, is insufficient to induce tumor formation (22,23). There are also functional and developmental differences between human and mouse OSE cells. In contrast to human, mouse OSE expresses high level of E-cadherin and considered to be terminally differentiated (24).

Here, we hypothesized that mice hemizygous for E-cadherin [*Cdh1*(+/-)] and lacking Msh2 will have high susceptibility for development of epithelial cancers, gynecologic in particular, due to progressive loss of second *Cdh1* allele that is driven by mutator phenotype (1)

caused by MMR deficiency. Indeed, we found that high percent of *Msh2*(-/-)/*Cdh*(+/-) crosses developed endometrioid tumors and that E-cadherin expression was lost in these tumors due to alterations in transcription regulatory elements at the promoter of this gene. In addition, *Msh2*(-/-)/*Cdh*(+/-) developed more aggressive than their *Msh2*(-/-) counterparts lymphomas with high proportion of these being B-cell type, thus also suggesting a specific role of E-cadherin in lymphomagenesis.

Methods

Generation of E-cadherin-deficient mouse model

C57Bl/6 mice heterozygous for E-cadherin, *Cdh1*^{tm^{Kem}} (23) were purchased in Jackson Laboratories (Bar Harbor, ME) and C57Bl/6 mice knockout for *Msh2* protein [*Msh2*(-/-)] were gift from Dr R.Kucherlapati. To generate mouse deficient in *Msh2* and expressing low level of E-cadherin [*Msh2*(-/-)/*Cdh*(+/-)] the initial *Msh2*(+/-)/*Cdh*(+/-) crosses were back crossed with first generation *Msh2* full knockout mice (Figure 1A). *Msh2*(-/-)/*Cdh*(+/-) and *Msh2*(-/-) progeny were screened for the presence of targeted *Cdh1* and *Msh2* alleles by polymerase chain reaction (Figure 1B, ref. 23). The *Msh2*(-/-) and *Msh2*(-/-)/*Cdh*(+/-) taken for survival analysis and tumor assessment were produced in the same litter (Figure 1A), thus were matched in mutational background. The breeding *Msh2* colony was maintained as heterozygous (+/-) animals to avoid genomewide mutation accumulation.

Mouse genotyping

For *Msh2* screening, 50 ng of DNA was amplified in a reaction with primers specific (10 pmol) to the wild-type allele (5'-CCCTCCTGTTGAGCCATCTTA-3' and 5'-TTCGCTGCTGTCTCTGGAAT-3') or to the *Msh2*(-/-) allele (5'-GCCAGCTCATTCTCCACTC-3' and 5'-CCCTCCTGTTGAGCCATCTTA-3'). In both cases, *Taq* polymerase (Perkin Elmer, Norwalk, CT) was used in 10 mM Tris-HCl, 10 mM KCl, 0.2 mM deoxynucleoside triphosphate and 1 mM MgCl₂ (pH 8.9). The polymerase chain reaction products were resolved by electrophoresis on 1% agarose gel. Wild-type and mutant alleles were distinguished by the size of the product (180 and 300 bp, respectively, Figure 1).

For E-cadherin-targeted allele, DNA was amplified with specific primers 5'-AAACCTGATGGATGTGGGATG-3' (E-cadherin) and 5'-GGTTTTCCCAGTCACGACGTTG-3' (β gal primer) in the same buffer with exception of MgCl₂ which was omitted. A knockout allele was detected as a 1 kb product (Figure 1B). Mice homozygous for the *Cdh1*^{tm^{Kem}}-targeted mutation are known to die at the time of implantation (23), therefore, only heterozygous carriers were picked up by the screen.

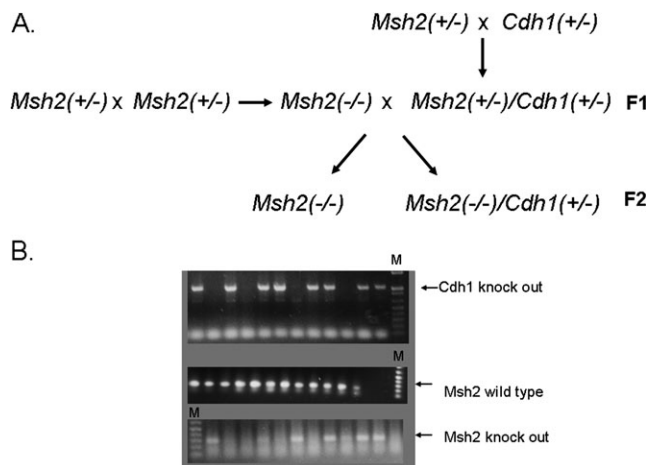


Fig. 1. Genotyping of generated *Msh2*(-/-)/*Cdh*(+/-) mice. *Msh2*(-/-) mice were crossed to *Cdh*(+/-) to generate *Msh2*(+/-)/*Cdh*(+/-) F1 progeny which subsequently were back crossed to *Msh2*(+/-) to generate F2 progeny that included *Msh2*(-/-) mice and mice deficient in *Msh2* and expressing low level of E-cadherin (*Msh2*(-/-)/*Cdh*(+/-)). The genotypes were confirmed by polymerase chain reaction using specific primers. Screening for the mutant (designated as knockout) and wild-type alleles of *Msh2* gene and mutant (designated as knockout) allele for *Cdh* gene was performed. Representative gels are shown.

Histopathological analysis

Mice were killed when found moribund or when showing signs of discernible tumors, tissues and tumors were collected, frozen or embedded in paraffin for histologic analysis and immunohistochemistry. For histology, 10 μm sections were stained with hematoxylin and eosin according to the standard procedures. For immunohistochemical analysis, the tumor sections were stained with antibodies for immunotyping, B lymphocyte-CD45R/B220 (cat# 553085; BD Pharmingen, San Diego, CA) and T lymphocyte-CD3 (cat# 14-0032; eBioscience, San Diego, CA) or antibodies directed against E-cadherin (cat #13-1900; Invitrogen, Camarillo, CA) and β-catenin (cat. # ab2982; Abcam, Cambridge, MA).

Immunoblotting

Whole cell extracts were prepared from frozen tissues according to standard procedure, and 40 μg of protein of each cell lysate was separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel. Protein was transferred onto polyvinylidene difluoride membranes, and the membranes were subsequently incubated with antibodies directed against E-cadherin and β-catenin (as above).

Sequencing

DNA was isolated from paraffin-embedded tissues either normal or tumor (characterized histologically). Sections of 10 μm were cut, deparaffinized by incubation in xylene for 15 min, washed with 100% ethanol and tissue pellet was subjected to lysis in the buffer containing Proteinase K. Extracted DNA was amplified using specific primers for promoter region of E-cadherin: forward 5'-ACGTGCGTTGCGAGCACACA-3', reverse 5'-AGAGGTCGCGCCAGGTGA-3' or 5'-TCCACACACAGTGAGCAGC-3'. The products were subsequently sequenced using Mayo Genomic facility and the sequence analysis was performed using MacVector and Sequencher software. To identify mutations in response elements, the sequences obtained from amplification products were aligned with sequence accession # NT_078575.6 and #AY566874 in the National Center for Biotechnology Information GenBank.

Statistical analysis

Time of death, body weight, gross pathology and tumor spectrum were recorded for each *Msh2*(-/-) and *Msh2*(-/-)/*Cdh*(+/-) mouse. The survival curves were generated using KaleidaGraph 3.6 software. The statistical differences between *Msh2*(-/-) and *Msh2*(-/-)/*Cdh*(+/-) curves were determined using log-rank test.

Fisher's exact test was used to determine statistical differences in tumor incidence between *Msh2*(-/-) (21 total analyzed) and *Msh2*(-/-)/*Cdh*(+/-) (20 total analyzed) mice.

Results

Tumor formation and survival

Alterations in E-cadherin expression play a significant role in initiation and progression of gynecological tumors. However, no satisfactory mouse model based on knockout of E-cadherin gene has previously been developed. Likewise, deficiency in MMR is implicated in tumorigenesis of solid tumors including ovarian and uterine, but knockout of MMR in mice predominantly causes hematologic tumors. Here, we set out to induce formation of gynecological tumors in MMR-deficient mice taking advantage of haploinsufficiency for E-cadherin. We hypothesized that *Msh2*(-/-)/*Cdh*(+/-) crosses will be prone to develop gynecologic tumors due to a progressive loss of second *Cdh1* allele. We bred *Msh2*(+/-) mice to *Msh2*(+/-)/*Cdh*(+/-) mice to generate *Msh2*(-/-)/*Cdh*(+/-) mice and mice deficient in *Msh2* and expressing low level of E-cadherin (*Msh2*(-/-)/*Cdh*(+/-)) (Figure 1A). Mice homozygous for the *Cdh1*^{tm^{Kem}}-targeted mutation are not viable while heterozygous *Cdh*(+/-) mice appear normal and do not show cancer predisposition (23). We monitored *Msh2*(-/-)/*Cdh*(+/-) crosses for cancer incidence and survival. When compared with *Msh2*(-/-) littermates *Msh2*(-/-)/*Cdh*(+/-) mice showed a reduced survival, with only 50% of animals surviving 16 weeks [26 weeks for *Msh2*(-/-), Figure 2A]. All *Msh2*(-/-)/*Cdh*(+/-) mice died by the age of 45 weeks [56 weeks in *Msh2*(-/-)]. Since the *Msh2*(-/-) and *Msh2*(-/-)/*Cdh*(+/-) mice were produced in the same litter and had similar mutational background (Figure 1A), we attributed the reduction in survival in the latter to deficiency in E-cadherin.

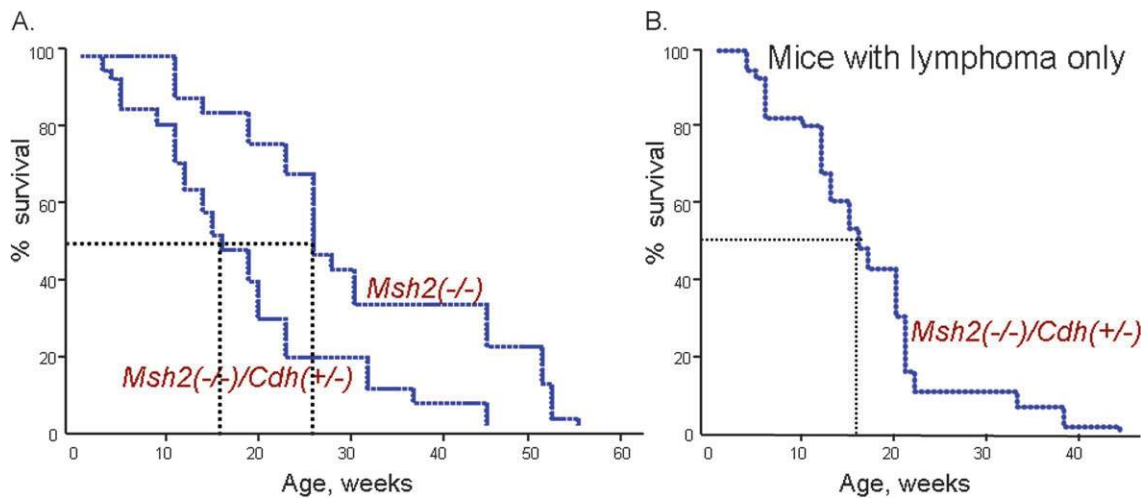


Fig. 2. Survival analysis for *Msh2(-/-)Cdh(+/-)* and *Msh2(-/-)* mice. (A) The survival of the *Msh2(-/-)Cdh(+/-)* (total of 50, included all that developed lymphomas and epithelial tumors) and *Msh2(-/-)* (total of 25) mice was compared. The difference between the two curves is significant as determined by log-rank test analysis ($P < 0.01$). (B) The survival of the *Msh2(-/-)Cdh(+/-)* which developed lymphomas only. Dashed lines show 50% survival in each chart.

Of 50 total *Msh2(-/-)Cdh(+/-)* mice in the survival analysis, 28 have shown visible tumors macroscopically. Complete histopathological analysis performed in 20 mice revealed that 65% had lymphomas and 35% other tumors (Table I). A number of mice showed generalized lymphomas involving multiple tissues. Two of twenty mice (10%) have developed both lymphomas and epithelial carcinomas (Table I). Surprisingly, we observed little neoplastic changes in the colon of *Msh2(-/-)Cdh(+/-)* mice (Table I). The spectrum of tumors in *Msh2(-/-)Cdh(+/-)* mice was significantly different ($P < 0.05$, determined by Fisher's exact test) from that in *Msh2(-/-)* counterparts. Twenty one *Msh2(-/-)* animals were included in the tumor assessment analysis, of which 17 (81%) developed tumors. Vast majority (94%) showed lymphomas, and one mouse had developed mucinous adenocarcinoma in the colon (Table II). The tumors appeared to be more aggressive in *Msh2(-/-)Cdh(+/-)* crosses as these mice had significantly shorter life span compared with *Msh2(-/-)* counterparts (Figure 2A). When *Msh2(-/-)Cdh(+/-)* animals with epithelial tumors were excluded from the survival analysis, the Kaplan-Meier curve did not change significantly, showing 50% of the animals surviving past 16 weeks (Figure 2B). Thus, the data suggest that more aggressive course of lymphomas in *Msh2(-/-)Cdh(+/-)* mice account for the reduced survival.

Increased incidence of endometrioid cancers in Msh2(-/-)Cdh(+/-) mice is due to aberrations in E-cadherin expression

Twenty percent of *Msh2(-/-)Cdh(+/-)* mice developed endometrioid-like tumors in the ovary, uterus and genital area (Table I). Endometrioid features of these tumors included a complex glandular structure and a stroma typical for endometrioid carcinoma (Figure 3A and supplementary Figure 1 is available at *Carcinogenesis* Online). One animal has developed both undifferentiated carcinoma in the uterus and endometrioid carcinoma in the ovary. Two of four mice that developed endometrioid tumors with no signs of lymphoma lived past 42 weeks of age.

We tested for correlation between E-cadherin expression and development of endometrioid tumors. No E-cadherin (Figure 3B) was detected in the uterine tumor (Figure 3A). Only mast cells were stained positively for E-cadherin in the uterus (Figure 3B, shown by arrow). Little of β -catenin staining was detected (Figure 3C). In contrast, normal uterus (Figure 3D and E) and normal ovary (supplementary Figure 2 is available at *Carcinogenesis* Online) showed a prominent staining for both proteins. Comparison of E-cadherin immunostaining in endometrioid abdominal tumor and in the uterus of the same mouse that displayed neoplastic changes side-by-side demonstrated loss of E-cadherin expression in both tissues (supplementary Figure 3A and B is available at *Carcinogenesis* Online).

Table I. Spectrum of tumors observed in *Cdh(+/-)Msh2(-/-)* mice

Tumor	<i>Cdh(+/-)Msh2(-/-)</i>
Lymphoma ^{a*}	13 (65%)*
Low grade	4 (20%)
High grade	9 (45%)
Endometrioid adenocarcinoma (ovary, uterus)	4 (20%)
Undifferentiated carcinoma (uterus, genital area)	2 (10%)
Anal squamous cell carcinoma	1 (5%)
Salivary gland adenocarcinoma	1 (5%)
Colon adenocarcinoma	1 (5%)

^aWide spread lymphoma was present in eight mice (40%). The asterisk indicates statistically significant differences in the number of specific tumors as judged by Fisher's exact test ($P < 0.05$).

Table II. Spectrum of tumors observed in *Msh2(-/-)* mice

Tumor	<i>Msh2(-/-)</i>
Lymphoma ^{a*}	16 (94%)*
Low grade	5 (29%)
High grade	11 (65%)
Mucinous adenocarcinoma (colon)	1 (6%)

^aWide spread lymphoma was present in five mice (29%). The asterisk indicates statistically significant differences in the number of specific tumors as judged by Fisher's exact test ($P < 0.05$).

We also performed immunoblotting analysis of expression of E-cadherin in a set of endometrioid tumors and uteri of *Msh2(-/-)Cdh(+/-)* and wild-type mice (Figure 3F and supplementary Figure 3C and D is available at *Carcinogenesis* Online). Total protein was extracted from frozen tissues and ECCD2 anti-E-cadherin antibody raised against N-terminus was used for detection. In some tumors (lanes 3 and 4, Figure 3F), E-cadherin was undetectable, indicating that the second allele was lost; in others, the level was significantly reduced (lane 1, Figure 3F) as compared with the level in wild-type uterus (Figure 3F, lanes 5 and 6). When quantified E-cadherin expression in the uterus of *Msh2(-/-)Cdh(+/-)* mouse with neoplastic changes (determined by histopathological analysis) was found lower than that in the uteri of *Msh2(-/-)Cdh(+/-)* without malignant transformation and wild-type mice (supplementary Figure 3C and D is available at *Carcinogenesis* Online).

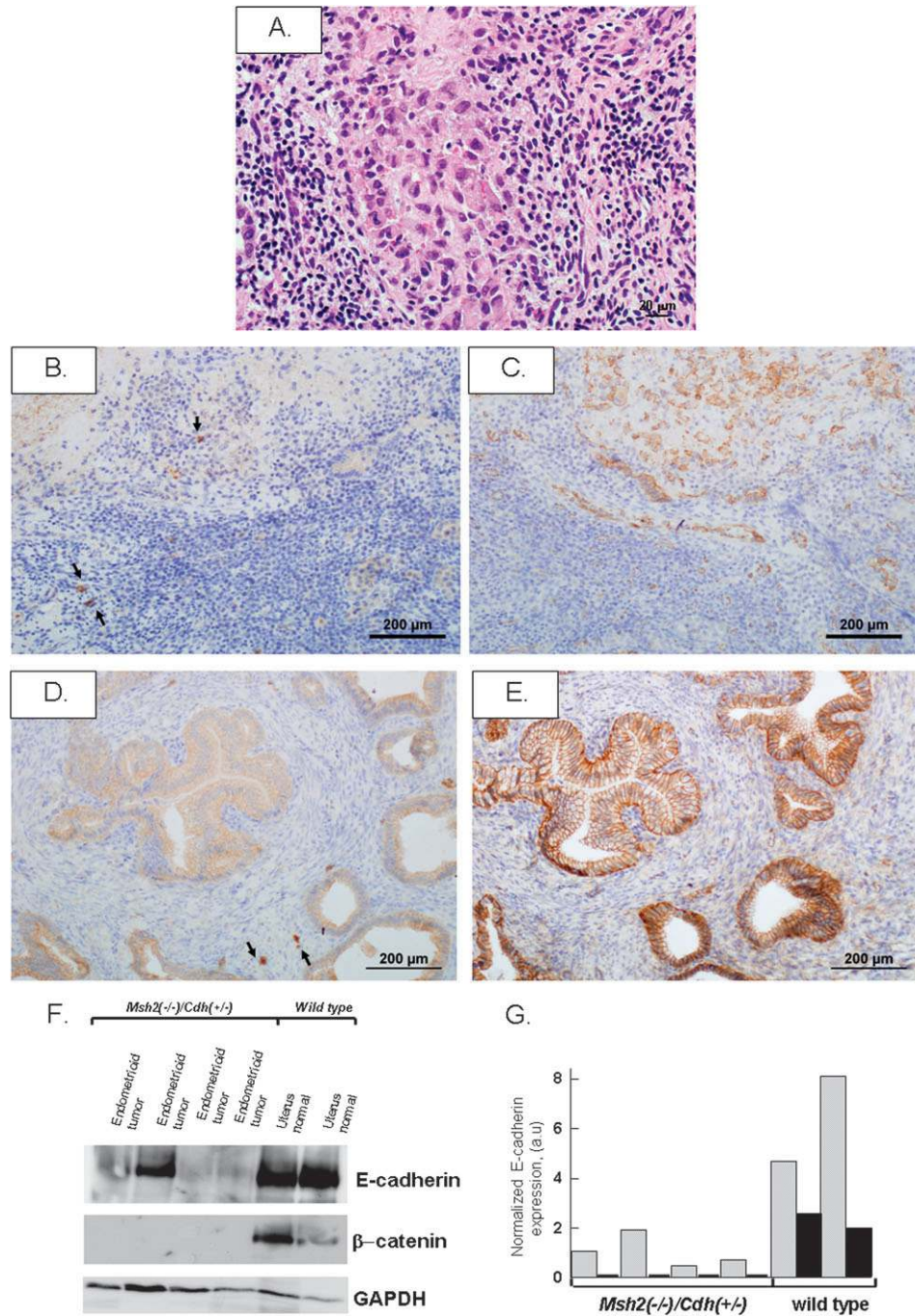


Fig. 3. Characterization of endometrioid tumors in *Msh2(-/-)/Cdh(+/-)* mice. (A) Hematoxylin and eosin staining of endometrioid tumor in uterus. Complex glandular formation and cytologic atypia are present ($\times 40$). (B and C) Immunostaining for E-cadherin and β -catenin, respectively, in the same tumor. Arrows in B show single E-cadherin-positive mast cells ($\times 20$). (D and E) E-cadherin and β -catenin in uterus of wild-type FVB mouse. (D) Immunostaining for E-cadherin. (E) immunostaining for β -catenin ($\times 20$). (F) Immunoblotting analysis of E-cadherin and β -catenin in mouse tissues. Protein extracts were obtained from indicated frozen tissues of *Msh2(-/-)/Cdh(+/-)* and wild-type mice. Antibody to detect glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used to verify equal loading. (G) Quantification of E-cadherin and β -catenin expression based on immunoblotting shown in F. The expression was normalized to the level of GAPDH. Gray bars and black bars represent E-cadherin and β -catenin, respectively.

Not all of analyzed epithelial tumors showed a reduction in E-cadherin expression beyond the level of single allele. In some tumor cases, we observed a number of cleavage products, including 97 and 80 kDa fragments as well as full-length 124 kDa protein (Figure 4, left panel lane 1 and right panel). Although there were no malignant changes in the colon tissue, we have also observed E-cadherin cleavage products (lane 2, left panel), similar to some endometrioid tumors. Soluble fragments of E-cadherin are suggested to interfere with

E-cadherin interactions, thus affecting normal cell adhesion, cell migration and intracellular signaling through β -catenin (25,26). Cleavage products of 97 and 80 kDa specifically have been reported to reduce adhesion function of E-cadherin and disrupt the β -catenin signaling (25,26). The role of the cleavage products in the genesis or progression of a particular tumor may also be tissue specific.

Loss of tumor suppressor PTEN is also frequently observed in endometrial carcinomas (19). We, therefore, examined PTEN expression

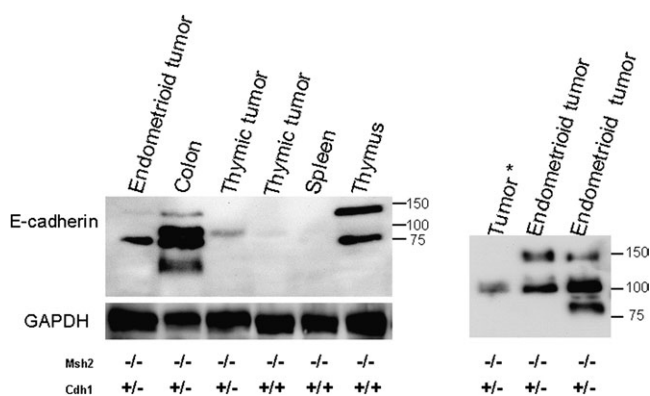


Fig. 4. Immunoblotting analysis of E-cadherin in mouse tissues. Protein extracts were obtained from indicated frozen tissues of *Msh2*(-/-)/*Cdh1*(+/-) and *Msh2*(-/-) mice. The genotypes are as shown. Mouse-specific antibody ECCD2 to N-terminus of E-cadherin were used. Antibody to detect glyceraldehyde-3-phosphate dehydrogenase were used to verify equal loading. The asterisk indicates undifferentiated carcinoma.

level in endometrioid tumors of *Msh2*(-/-)/*Cdh1*(+/-) mice. In contrast to E-cadherin, the level of PTEN was not different in tumors as compared with uterine tissue of wild-type mice (data not shown).

Mutational analysis of E-cadherin promoter

Silencing of E-cadherin expression in human cancers has been described as a result of promoter methylation, inhibition by transcriptional repressors and epigenetic silencing by the polycomb complex (14–16,27). A number of cis-acting regulatory transcription elements have been identified in E-cadherin promoter region (28). GC-rich sequences located at -60 to -23 nt are responsible for basic epithelial promoter activity and contain binding sites for SP1 transcription factors (28). E-pal region comprised of sequences between -75 and -88 nt was shown to contain repressor elements (28).

To examine whether a loss of expression of E-cadherin in epithelial tumors of *Msh2*(-/-)/*Cdh1*(+/-) mice was due to mutations caused by *Msh2* deficiency and accumulated in the promoter region, we sequenced 300 bp upstream of transcription start site of the E-cadherin gene. DNA from lymphoid and epithelial tumors was analyzed. No mutations were detected in E-pal region or CAAT box in any of the analyzed tissues. In contrast, we found that CG-rich region (Figure 5A) was often mutated in tumors of *Msh2*(-/-)/*Cdh1*(+/-) mice. All four endometrioid tumors were sequenced, three that showed loss of expression of E-cadherin (Figure 3) harbored mutations in CG-box. In addition, one of undifferentiated carcinomas with no expression of E-cadherin also showed mutations in CG-box (Figure 5B). The observed mutations were within the subregions of CG-box that contained binding sites for SP1 and AP2 and have been previously demonstrated to knockdown promoter activity in functional reporter gene experiments (28). Thus, in a subset of epithelial tumors, the mutations in CG-rich region (Figure 5B and C) correlated with the loss of E-cadherin expression (Figure 5), suggesting that a decrease in promoter activity of the remaining E-cadherin allele accounts for the lack of the protein.

T-cell to B-cell type lymphoma shift in *Msh2*(-/-)/*Cdh1*(+/-) mice

Lymphomas have been described previously in 70–75% of *Msh2*(-/-) mice and were reported to be T cell only. We examined *Msh2*(-/-)/*Cdh1*(+/-) lymphomas (Figure 6A and B) for expression of T-cell- and B-cell-specific markers. While majority of lymphoma tumors remained T-cell type, strong B220-positive phenotype was found in 3 of 13 (25%) assessed lymphomas (Table I, Figure 6D–F). Lymphoblastic T-cell lymphomas, diffuse large B-cell lymphomas and Burkitt lymphomas, classified according to the Bethesda proposal for lymphoid neoplasms in mice (29) were identified in *Msh2*(-/-)/*Cdh1*(+/-) animals (Figure 6).

The tumor tissues were also stained with antibody to E-cadherin and β -catenin (Figure 6G and H). The analyzed slides showed no or little staining for E-cadherin (Figure 6G). The rare weak staining for E-cadherin in a few cases corresponded to cells other than lymphocytes. A few positive for β -catenin lymphocytes with membrane staining were typically observed in lymphomas (Figure 6H).

The staining in tumors of *Msh2*(-/-)/*Cdh1*(+/-) for these two markers did not differ from that in lymphomas of *Msh2*(-/-) mice (data not shown). Similarly, human diffuse large B-cell lymphoma cells were negative for E-cadherin staining and mostly negative for β -catenin (supplementary Figure 4 is available at *Carcinogenesis* Online).

Consistent with immunostaining (Figure 6), little if any expression of E-cadherin was detected in thymic tumors by immunoblotting in either *Msh2*(-/-)/*Cdh1*(+/-) or *Msh2*(-/-) mice (Figure 4).

We attribute the reduction in survival of *Msh2*(-/-)/*Cdh1*(+/-) to haploinsufficiency of E-cadherin. The mechanism by which lymphomas disseminate faster is not clear, as normal mature lymphocytes are believed not to express E-cadherin.

Discussion

We reported here that mice with the *Msh2*(-/-)/*Cdh1*(+/-) genotype were prone to development of endometrioid tumors (Table I). Tumor development was associated with loss of expression of E-cadherin and with accumulation of mutations in the promoter region of *Cdh1* gene.

In humans, defects in MMR and loss of E-cadherin are linked independently to development of gynecological cancers. Somatic loss of heterozygosity at a specific gene is often considered to be a trigger for tumorigenesis. In contrast, mice heterozygous for MMR proteins or E-cadherin are not prone to cancer, possibly due to a short life span, but a homozygous knockout for an MMR protein is associated with high susceptibility to lymphomas (8). A number of recent studies have shown a similar trend in patients with germ line mutations in both copies of any one of the MMR genes (30,31). These mutations were found to be associated with an early onset of childhood T- or B-cell leukemias (31,32). While generally considered not to be HNPCC related, a few cases of lymphomas and leukemias were also observed in certain kindreds of HNPCC (33). Murine MMR-deficient lymphomas were shown to represent homogenous histopathologic type (34), suggesting that these tumors arise by a common pathway. The search for mutations in genes contributing to tumorigenesis of lymphoma has identified inactivating insertion/deletion mutations at the mononucleotide repeats of transforming growth factor- β receptor type II in tumors of *Msh2*(-/-) knockout mice (35). In another study, Ikaros, a key protein in lymphoid lineage differentiation, implicated in T-cell lymphomagenesis in humans, was shown to be a mutational target in Mh1-deficient mouse T-cell lymphomas (36). In the present work, we hypothesized that E-cadherin gene is another site that is frequently mutated in the absence of functional MMR and that complete loss of this protein increases incidence of gynecological cancers. In fact, we discovered that 20% of *Msh2*(-/-)/*Cdh1*(+/-) developed endometrioid-like tumors in the ovary, uterus and genital area (Table I, Figure 3). Although these tumors had similar histopathological features, the site of their origin is probably to be different in each case. In humans, the cell-of-origin for OC remains an unresolved issue. OSE is one of the sites where OC is considered to originate. The support comes from histopathologic evidence, developmental analysis (37) and experimental models (38). The alternative hypothesis of site of origin for OC suggests that it arises in distal oviduct and later metastasizes to the ovary. This hypothesis is supported by the discovery that dysplastic lesions and *in situ* carcinomas are present in fimbriated end of the fallopian tube and not in OSE in high percent of OC cases (39). EC is thought to be initiated in epithelial progenitor cells, which reside in normal endometrium. They have been isolated and shown to have both self-renewal and tumorigenic properties (40). Aberrations in E-cadherin/ β -catenin pathways play a role in tumorigenesis of both OC and EC. Changes in expression and presence of inactivating/activating mutations in both E-cadherin and β -catenin are documented in serous and endometrial types of OC (41,42). Inactivation

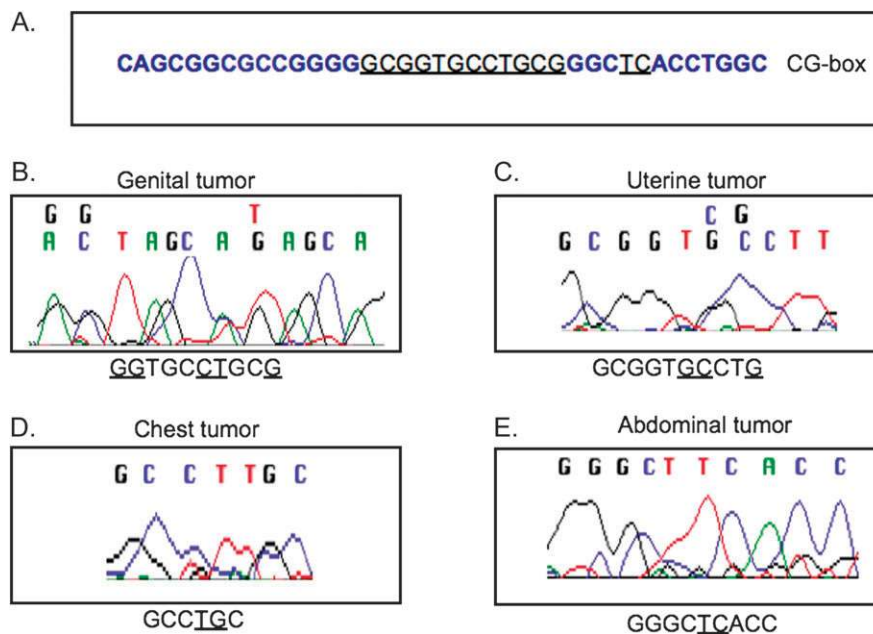


Fig. 5. Sequence analysis of the E-cadherin promoter in tumors of *Msh2*($-/-$)/*Cdh*($+/-$) mice. (A) Sequence of CG-box (-60 to -23 nt) is shown. Underlined nucleotides correspond to those found mutated in tumors of *Msh2*($-/-$)/*Cdh*($+/-$) mice. (B–E) Representative chromatograms showing mutations in CG-box in depicted tumors, endometrioid (B and C) and lymphomas (D and E). The correct sequences are shown at the bottom of each chromatogram, mutated nucleotides are underlined.

of E-cadherin is shown in 80–90% of the cases of high-grade EC characterized by a progressive behavior and deep myometrial invasion (19). Furthermore, expression analysis in endometrial serous carcinoma suggested that loss of β -catenin contributes to its pathogenesis and progression (43). Analogous to OC, defects in MMR have also been reported in EC cases (32). Our data from *Msh2*($-/-$)/*Cdh*($+/-$) mice are not conclusive to suggest whether OSE, fallopian tube or uterus is the site of origin of endometrioid tumor in each case. Further experiments using our *Msh2*($-/-$)/*Cdh*($+/-$) model are needed to address the issue of origin.

Our analysis of E-cadherin cleavage products and gene promoter suggests potential mechanisms underlying development of endometrioid tumors in *Msh2*($-/-$)/*Cdh*($+/-$) mice. A complete loss of E-cadherin (Figure 3) driven by *Msh2* deficiency was mainly due to mutations at transcription elements in the E-cadherin promoter region (Figure 5). In the tumors where expression of E-cadherin was preserved, we observed a number of cleavage fragments (Figure 4). E-cadherin is a known target for proteases, a number of which have been reported to be overexpressed in cancer. Soluble fragments of E-cadherin are thought to interfere with E-cadherin interactions thus affecting normal cell adhesion, cell migration and intracellular signaling through β -catenin (25,26). Some E-cadherin cleavage products have been linked to the malignant progression of adenocarcinomas. Further studies are needed to confirm the significance of cleavage fragments we observed in endometrioid tumors of *Msh2*($-/-$)/*Cdh*($+/-$) mice. One explanation for the presence of cleavage fragments is that MMR deficiency induces mutations that lead to elevated expression of genes coding for proteases, which target E-cadherin.

The spectrum of tumors observed in various knockout for MMR enzymes mice was shown previously to shift with the introduction of additional genetic alteration. *Msh2*- and *Msh6*-deficient mice bred to athymic nude mice have developed B-cell lymphoblastic lymphomas (34). MMR-null mice crossed with animals bearing point mutation in *Apc* protein (*Apc*1638N) showed increased intestinal tumor incidence and reduced survival (44). Double mutant *Pten*($+/-$)/*Mlh1*($-/-$) mice showed accelerated compared with *Pten*($+/-$) mice endometrial tumorigenesis that was attributed to a loss of the wild-type *Pten* allele (45).

We did not observe a complete shift from lymphoma to epithelial tumors in *Msh2* knockout when crossed with *Cdh1*($-/+$). The results

suggest that the remaining allele of E-cadherin if not inactivated somatically is sufficient to prevent neoplastic transformation in epithelial cells.

Msh2($-/-$)/*Cdh*($+/-$) mice that we generated show a significant reduction in survival as compared with *Msh2*($-/-$) counterparts (Figure 2) and a shift from T-cell to B-cell type lymphoma (Figure 6). The role of E-cadherin in hematologic malignancies is not entirely clear. E-cadherin is believed not to be expressed in mature lymphocytes. The E-cadherin level, however, is high in immature thymocytes in fetus, and thymic epithelial cells (46). Homotypic E-cadherin adhesion was noted to be important for association of thymic epithelial cells and subsequent thymocyte development in fetal thymus (46). Similarly, E-cadherin expression was detected in erythroblasts and normoblasts in bone marrow but not in mature erythrocytes (47). In bone marrow and peripheral blood samples of acute myelogenous leukemia and chronic lymphocytic leukemia patients, the level of E-cadherin was reduced or undetectable as compared with normal counterparts. Loss of expression was due to hypermethylation of the gene promoter, similar to solid tumors (48). In more recent study, inactivation of E-cadherin gene due to aberrant splicing has been discovered (49). In chronic lymphocytic leukemia, the accumulation of nonfunctional aberrantly spliced transcripts was accompanied by decreased expression of E-cadherin and activation of Wnt signaling.

In mouse primary thymic lymphomas, E-cadherin expression in adjacent stromal cells was reduced, and the reduction in expression occurred concomitantly with the progression of lymphomas (50). Thus, perturbations in microenvironment apparently contribute to development of thymic tumors. Furthermore, canonical and non-canonical Wnt- β -catenin-Tcf pathways have been implicated in normal hematopoiesis (51) and pathogenesis of several hematologic malignancies (52–54). For example, hematopoietic stem cells in β -catenin-deficient mice are impaired in a long-term growth and maintenance following transplantation (51). On the other hand, activation of β -catenin enhances self-renewal of hematopoietic stem cells *in vitro* and *in vivo* (51). Tcf/Lef1 and its target genes are upregulated in chronic lymphocytic leukemia (52), activated in mantle cell lymphoma and chronic myeloid leukemia (53,54).

Although, we did not observe any differences in expression of E-cadherin or β -catenin in lymphomas of *Msh2*($-/-$)/*Cdh*($+/-$)

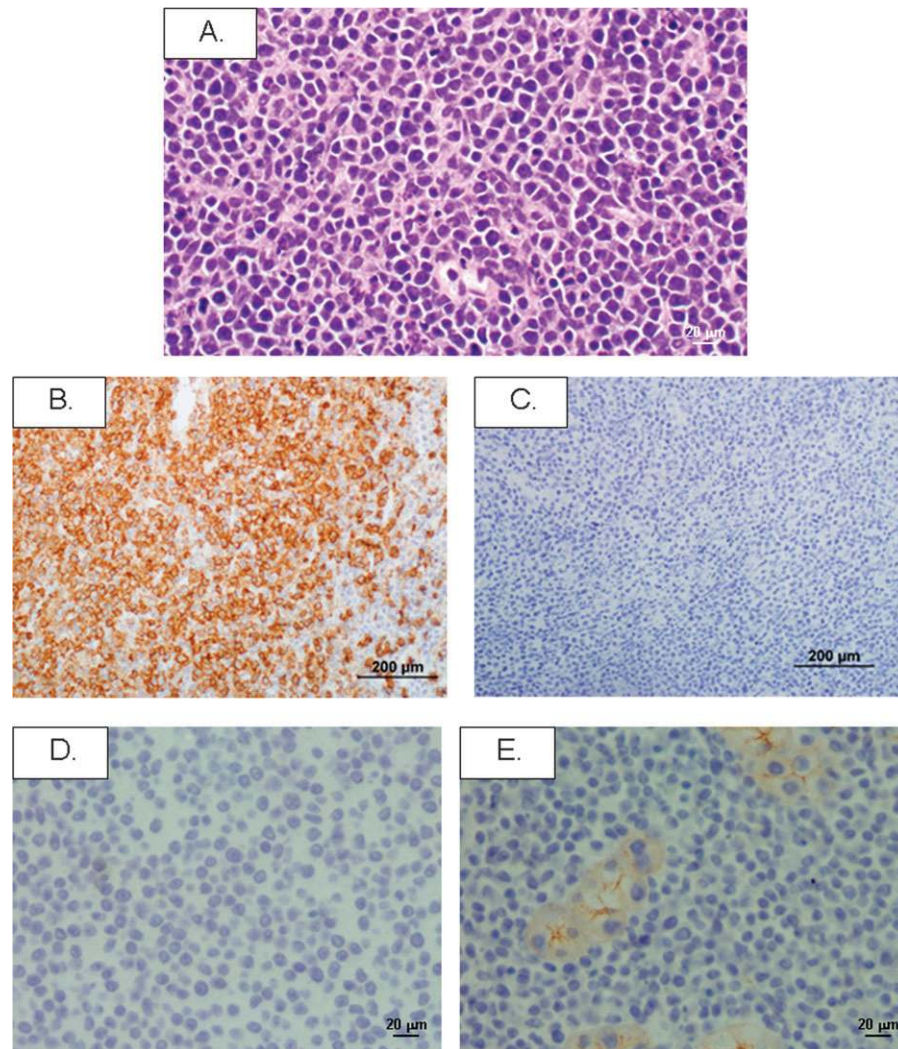


Fig. 6. Characterization of lymphoma in *Msh2*($-/-$)/*Cdh*($+/-$) mice. (A) Hematoxylin and eosin staining of large cell lymphoma. Large cells with high nuclear-to-cytoplasmic ratio and occasionally prominent nucleoli. Mitotic activity is evident ($\times 40$). (B and C) Immunostaining for lymphocyte-specific antigens. The cells show B-cell phenotype (B) as they are negative for CD3 (C) ($\times 20$). (D and E) Immunostaining for E-cadherin (D) and β -catenin (E) in the same tumor as in A–C ($\times 40$). All sections are counterstained with hematoxylin.

versus *Msh2*($-/-$) mice, our finding that lymphomagenesis in the former is accelerated and shifted toward B-cell type points to a specific yet to be elucidated role of E-cadherin in the progression of lymphomas. One of the possibilities is that a decrease in expression of E-cadherin (due to mutations in the promoter region) in hematopoietic stem cells sets off a cascade of changes in Tcf/Lef1- β -catenin pathway which subsequently lead to a promotion of lymphomagenesis. Alternatively, reduction in expression of E-cadherin in surrounding tumor stromal cells may facilitate tumor growth and/or tumor dissemination. This mouse model, therefore, can serve to complement human studies to clarify the role that E-cadherin plays in hematopoiesis and hematologic malignancies.

Finally, since both MMR (55,56) and E-cadherin (57,58) are implicated in tumor response to DNA-damaging drugs used to treat lymphomas and gynecological cancers in humans, the *Cdh1*($-/+$)/*Msh2*($-/-$) mouse model can provide a useful tool to test therapies that are tailored toward groups of patients deficient in MMR and E-cadherin.

Supplementary material

Supplementary Figures 1–4 can be found at <http://carcin.oxfordjournals.org/>

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