Supplementary 1

Possible hereditary gastric cancer revealed by genetically engineered mice and family history of hereditary breast and ovarian cancer (HBOC)

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Material and Methods

Produce of Gan^{tgBrca1} and Gan^{tgBrca2}

MMTV-BRCA1 transgenic constructs: Diagram of the *BRCA1* cDNAs used for the generation of transgenic animals. The expression of wild type *BRCA1* is controlled by the MMTV-LTR promoter. The *BRCA1* cDNAs were each inserted into the third exon of the rabbit *ß-globin* gene (*ß-g*). The bar indicates the RING finger motif, the hatched region corresponds to the nuclear localization signals and the negative symbols (-) indicate the negatively charged C-terminal domain with transactivation function.

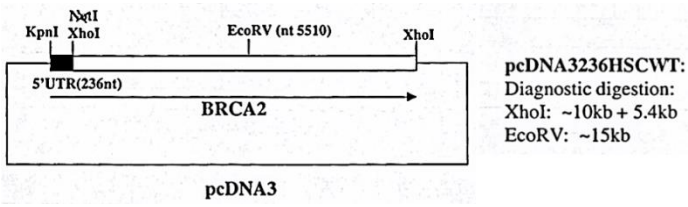
Generation and Maintenance of BRCA1 Transgenic Mice: The Xho I BRCA1 fragments were microinjected into the male pronucleus of (C57BL/6x DBA/2) F1 fertilized mouse embryos and implanted into pseudopregnant ICR surrogate mice by the Transgenic/ES Cell Shared Resource Facility. Founder mice were bred to C57BL/6J mice to establish transgenic lines. All animals used in these studies were handled in strict compliance with Shinshu University School of Medicine, Animal Care Committee regulations.

To identify transgenic animals by PCR, 1µL of genomic tail DNA was added to a 50µL reaction volume [1x PCR buffer II, 1.5mM $MgCl_2$, 200nM each of rabbit *ß-g* forward 5'-GTCTCGGAT CCTCAGAAGGTGGTGGCTGGTGTGG-3' and rabbit *ß-g* reverse 5'-GAACTAGGTACCGGCAT ATGTTGCCAAACTCTAAACC-3' primers, 200µM each dNTP, and 2.5 units of AmpliTaq DNA polymerase (Perkin-Elmer)]. Cycling conditions consisted of 94ºC for 6 min, followed by 25 cycles of 94ºC for 30 sec, 64ºC for 60 sec, and 72ºC for 90 sec, and a final extension at 72ºC for 3 min. The PCR product was a 260bp fragment of the rabbit *ß-g* exon 3 contained in the transgene construct.

The copy number of founder transgenic animals was estimated by determining the amounts of DNA equal to one copy of the integrated transgene. The approximate number of base pairs of the promoter and transgene injected (8400bp for MMTV-BRCA1) was divided by the approximate size of the mouse haploid genome (3 x 10^9 bp). Thus, 2.8pg of the promoter/transgene fragment is equal to approximately one copy of the integrated MMTV-BRCA1 transgenes per 1µg of genomic DNA, while 1.3pg is equal to approximately one copy of the integrated MMTV-BRCA1t340 transgene per 1µg of genomic DNA. The relative intensity of probe hybridization was compared using a Bio-Rad Model GS 670 Imaging Densitometer.

CMV-BRCA2 transgenic constructs: To construct p236BRCA2, the pcDNA3 vector was first modified by inserting a 236-bp fragment of the 5' untranslated region of BRCA2 between the KpnI and NotI sites. The assembled full-length BRCA2 cDNA was then inserted at the XhoI site of this plasmid. The 5' UTR of BRCA2 was obtained by RT-PCR using primers 5'-GGTACCGGTG GCGCGAGCTTCTGA-3' and 5'-GCGGCCGCAACTACGATATTCCTCCAAT-3'. The pcDNA3 236HSC WT (BRCA2) was a gift from Mien-Chie Hung (Addgene plasmid # 16246; http://n2t.net/addgene: 16246 ; RRID:Addgene_16246) (I)

Generation and Maintenance of BRCA2 Transgenic Mice: The *BRCA2* was generated using recombineering, and transgenic mice were generated as described previously (II). All animals used in these studies were handled in strict compliance with Shinshu University School of Medicine, Animal Care Committee regulations.



Gan^{tgBrca1} and **Gan**^{tgBrca2} mice were created by crossing C57BL/6J^{tgBrca1} or C57BL/6J^{tgBrca2} mice with Gan mice (provide from Dr. Oshima M, Kanazawa University School of Medicine, Kanazawa, Ishikawa, Japan). Gan mouse is a compound transgenic mouse created by crossing C2mE mouse and Wnt mouse. In Gan mice, both the COX-2/PGE2 pathway and Wnt signaling are activated in the gastric mucosa. Gan mice spontaneously develop ductal gastric cancer accompanied by an inflammatory response with 100% efficiency. Gan mice are considered to be a model similar to human gastric cancer, where tumors develop through the interaction of Wnt signal activation and inflammatory responses. All animals used in these studies were handled in strict compliance with Shinshu University School of Medicine, Animal Care Committee regulations (Approved number: Shinshu University 567-5).

Produce of Gan mouse:

Gan mice were created by crossing *K19-Wnt1* transgenic mice with *K19-C2mE* mice (provide from Dr. Oshima M, Kanazawa University School of Medicine, Kanazawa, Ishikawa, Japan) (III). The K19 promoter with a synthetic intron and SV40poly(A) cassette were described previously.6 Although the K19 promoter used in the present study is transcriptionally active in the gastric epithelium, its expression spectrum in the whole body is slightly different from the endogenous K19 gene, possibly caused by the limited length of the promoter fragment (III). *Wnt1* complementary DNA (cDNA) was excised from pUSEamp-Wnt1 (Upstate, Charlottesville, VA). These fragments were cloned into pBluescript (Stratagene, La Jolla, CA) to construct the pK19-Wnt1 transgenic vector. The expression vector was microinjected into the fertilized eggs of F1 (C3H and C57BL/6J) hybrid females crossed with C57BL/6 males to generate *K19-Wnt1* transgenic mice. Expression of *Wnt1* in the gastric mucosa was confirmed by reverse-transcription polymerase chain reaction (RT-PCR). Construction of *K19-C2mE* transgenic mice has been described previously. To minimize any genetic background differences, we used littermate mice for the experiments from the mating of N2-backcrossed *K19-Wnt1* with N6-backcrossed *K19-C2mE* mice. Backcrossing was performed using wild-type C57BL/6 mice. Construction of *Apc_716* mice has been previously described.2 All animal experiments were carried out according to the protocol approved by the Ethics Committees on Animal Experimentation of Shinshu University.

The expression vector was microinjected into the fertilized eggs of F1 (C3H and C57BL/6) hybrid females crossed with C57BL/6 males to generate K19- Wnt1 transgenic mice. Expression of Wnt1 in the gastric mucosa was confirmed by reverse-transcription polymerase chain reaction (RT-PCR). Construction of K19-C2mE transgenic mice has been described previously (III). A 2.1-kb promoter fragment K19 (GenBank, AF237661) amplified by genomic PCR, a 1.0-kb SV40 polyA cassette and a synthetic cliimerie intron excised from pCl (Promega, Madison, WI, USA) were cloned into pBluescript vector (Stratagene, La Jolla, CA, USA) to construct pK19. Full-length cDNAs for COX-2 and mPGES-1 were amplified by RT-PCR. After sequence confirmation, the eDNA fragments were subcloned into pK19 to construct pK19-COX-2 and pK19-mPGE5, respectively. Two expression vectors were co-micro- injected into fertilized eggs of the F, (C3 H and C57BL/6) hybrid females crossed with C57BL/6 male. Two of the six constructed transgenic lines, K19-C2mE-2 and *K19-*

C2inE-8 showed high expression levels of COX-2 and mPGES-1, and N2-backcrossed mice with CS7BL/6 of these lines were used for further analysis. Wild-type litterniates were used as controls.

Analysis of reverse transcription-PCR (RT-PCR) analysis: Total RNA was extracted from the glandular stomach and primary culture epithelial cells, using ISOGEN (Nippon Gene, Tokyo, Japan). Extracted RNA was reverse-transcribed and PCR-amplified. The isolation of total RNA from the different tissues of 5-6-mo old F1 PCR-positive offspring of founder (s), and non-transgenic littermates of F1 PCR positive transgenic pups and normal mice as negative controls was performed using the RNeasy Mini Kit (Qiagen) following the manufacturer's recommendations. Purified RNA was eluted in a final volume of 50 μL DNA-free water and aliquots were stored at -80 ℃ with 2 μL of RNasin. Extracted RNA was reverse-transcribed and PCR-amplified. Band intensities of the RT-PCR products were quantified in a densitometer, using Image I (NIH, Betliesda, MD, USA).
A specific system for the amplification of mRNA used was one-step mRNA selective PCR kit (version 1.1) (TaKaRa). RT-PCR was carried out as recommended by the manufacturer (Takara) with minor modifications. Briefly, it was carried out in a volume of 50 μL including 25 μL 2× mRNA selective PCR buffer I, 10 μL 25 mmol/L MgCl2, 5 μL 1 mmol/L dNTP/analog mixture each, 1 μL RNase inhibitor (40 U/mL), 1 μL AMV reverse transcriptase XL (5 U/mL), 1 μL AMV-optimized Taq (5 U/mL). In the reaction volume of 50 μL, 1 μg total RNA was used to synthesize the single-stranded cDNA with AMV reverse transcriptase XL (Takara) in one-step RT-PCR. The oligonucleotide primers used for RT-PCR were rtTA-FP/ RP primers (see above for details) (PCR product size: -1 kb). RT-PCR amplification was carried out as follows: 30 min at 50 ℃ for RT, denaturation for 5 min at 85 ℃ and then a succession of 35 cycles as follows: 1 min at 85 ℃, 1 min at 58 ℃, 90 s at 72 ℃, and a final extension at 72 ℃ for 10 min. Specific β-actin primers were used for the internal control to normalize the sample amounts. RT-PCR was carried out using the following the primer sets: COX-2 (F-5'-CAAACTCAAGTTTGACCCAG-3', R-5'-GCCGGGATCCTTTTACAGCTCAGTTGAACG-3'), tgBRCA1 (F-5'-GTCTCGGATCCTCAGAAGGTGGTGGCTGGTGTGG-3' R-5'-GAACTAGGTA CCGGCATATGTTGCCAAACTCTAAACC-3'), tgBRCA2 (F-5'-GGTACCGGTG GCGCGAGCTT CTGA-3' and R-5'-GCGGCCGCAACTACGATATTCCTCCAAT-3')

Immunostaining for detection of BrdU positive cells in tumors
Preparation of BrdU solution: Retrieve the BrdU solution from -20 ℃ freezer and allow it to equilibrate at room temperature (RT). Calculate the mass of BrdU needed for a dose of 50 mg/kg according to the body weight of the mouse. Calculate volume of 0.9% saline solution (0.9 g NaCl in 100 mL of sterile H_2O) needed for a working solution of 20 mg/mL. Prepare an excess to provide at least 0.5 mL per mouse per injection. NOTE: The dose administered to experimental animals should be safe, with minimal side effects, and effective. It has been reported that duration of staining with 100 mg/kg BrdU does not outweigh the potentially higher toxicity compared with the 50 mg/kg dose[7]. No significant differences were found in the number of BrdU-labeled cells/mm^3 for 50 and 100 mg/kg i.p. in mouse. It is preferable to inject a small dose to minimize the suffering of the animals. Weigh out BrdU solution and add it to the saline solution in a conical tube and vortex.

NOTE: BrdU solution is toxic and potentially carcinogenic. Prepare it in the fume hood. BrdU solution must be handled with proper protective equipment (PPE). It is recommended to prepare the solution immediately before use. However, the solution is stable for 24 h under RT. Please protect it from light.

To prepare 1 L of 0.1 M phosphate buffered saline (PBS) at pH 7.4, add 240 mg of potassium phosphate monobasic (KH_2PO_4), 1.44 g of sodium phosphate dibasic (Na_2HPO_4), 200 mg of potassium chloride (KCl), and 8 g of sodium chloride (NaCl) to 800 mL of double distilled water (ddH_2O) under constant stirring. Adjust the pH to 7.4 and add double distilled H_2O up to total volume of 1 L. Store at 4 ℃ for up to 1 week. For 100 mL of PBS+, add 3% (3 mL) of normal horse serum and 0.3% (300 μL) of Triton X-100 to 0.1 M PBS (pH 7.4). Store in 20–50 mL aliquots at -20 ℃ for

up to 3 months. For 100 mL of PBS++, add 10% (10 mL) of normal horse serum and 0.3% (300 µL) of Triton X-100 to 0.1 M PBS pH 7.4. Store in 20–50 mL aliquots at -20 °C for up to 3 months. For 1 L of cryoprotectant solution, mix 250 mL of ethylene glycol and 250 mL of glycerol, constantly stir until mixed. Slowly bring to 1 L with PBS. Filter with grade 4 (20–25 µm) filter paper. Store at 4 °C or RT for up to 1 year. Prepare 4% paraformaldehyde in 0.1 M PBS (PFA solution) as follows. For 1 L of solution, add 40 g of paraformaldehyde powder slowly to 800 mL of 60–65 °C 0.1 M PBS under constant stirring. Stir until paraformaldehyde is completely dissolved while controlling the temperature (60–65 °C). If necessary, add a few drops of 1 M NaOH to clarify the solution. When the solution reaches room temperature, filter with grade 4 (20-25 µm) filter paper. Paraformaldehyde is toxic and is suspected of being a carcinogen, prepare in the fume hood. Store at 4 °C for and preferably use within to 2 days. PFA ready-to-use solution is commercially available. For 1 L of 10 mM Sodium citrate buffer (SCB) at pH 6, add 1.204 g of sodium citrate (dihydrate), and 1.134 g of citric acid to 800 mL of double distilled H_2O under constant stirring. Adjust the pH to 6.0 and add ddH_2O up to 1 L. Store at 4 °C for up to 6 months. Prepare 50 mL of 2 N HCl by slowly adding 8.25 mL of 12 N HCl (concentrated stock solution) to 41.75 mL of ddH_2O under constant stirring. N HCl will be used for DNA denaturation, a crucial step. As BrdU is incorporated into the DNA, HCl is used to open the DNA bonds allowing BrdU antibody access BrdU within the DNA.

Prepare endogenous peroxidase blocking solution as follows. Prepare 100 mL of 0.6% hydrogen peroxide by mixing 2 mL of 30% hydrogen peroxide with 98 mL of ddH_2O under constant stirring. The solution must be prepared immediately before use. Keep it in the dark as H_2O_2 is light sensitive. PBS or TBS can be used instead of water. Prepare avidin-biotin complex (ABC) solution as per the instructions from the manufacturer. For 5 mL of ABC in 0.1 M PBS, add 2 drops (≈100 µL) of reagent A and mix, and then add 2 drops (≈100 µL) of reagent B and mix. Solution must be prepared and allowed to tumble-roll for 20–30 min before use. Prepare DAB (Diaminobenzidine) Peroxidase (HRP) substrate using the kit by following the instructions of the manufacturer. To 5 mL of ddH_2O, add 2 drops (≈ 84 µL) of reagent 1 and mix, add 4 drops (≈ 100 µL) of reagent 2 and mix, then add 2 drops (≈ 80 µL) of reagent 3 and mix. Finally, if desired, add 2 drops (≈ 80 µL) of reagent 4 (Nickel) and mix. DAB is toxic and potentially carcinogenic. It must be handled with care and discarded as per the hazardous waste regulation at each institution. To inactivate DAB, add several drops of bleach (sodium hypochlorite); the solution will turn black. Prepare 100 mL of cresyl violet solution by adding 100 mg of cresyl violet acetate and 250 µL of acetic acid to 80 mL of ddH_2O at 55–60 °C. Adjust the volume to 100 mL, filter, and store at 4 °C in a dark-colored vessel.

Thymidine analog BrdU administration: Restrain the experimental animal (e.g., 10-week-old male C57BL6J mouse weighing 24 g), by immobilizing the lower abdominal cavity. Administer the BrdU solution (50 mg/kg) intraperitoneally (i.p.) using a 23 G needle and 1 mL syringe.

Immunostaining: Detection of BrdU using peroxidase reaction with DAB. Transfer slices from the cryoprotection solution to 0.1 M PBS at room temperature. Rinse three times for 10 min each, with 0.1 M PBS. Incubate slices for 30 min in endogenous peroxidase blocking solution to inactivate endogenous peroxidase. Rinse 3 times, 10 min each, with 0.1 M PBS. Optionally, perform antigen retrieval (see section 5). Incubate slices for 20 min with 2 N HCl at 37 °C. Rinse in 0.1 M borate buffer (8.5 pH) for 10 min. Rinse 3 times for 10 min each, with ice-cold 0.1 M PBS. Incubate slices for 2 h at room temperature with PBS^{++} (blocking solution). Incubate with anti-BrdU primary antibody (mouse host) at concentration of 1:250 in PBS^{+} overnight at 4 °C. On day 2, rinse the slices 3 times for 10 min each with 0.1 M PBS. Incubate with 1:250 HRP-conjugated secondary antibody (anti-mouse) in PBS+ for 2–4 h at room temperature. Rinse 3 times for 10 min each with 0.1 M PBS. Transfer slices to DAB Peroxidase (HRP) Substrate solution and incubate for 2–10 min. When slices become dark grey, visualize the tissue with a microscope. If positive cells are present, rinse 3 times (for 15 min each) with tap water (to reduce background). Wash 3 times for 10 min each with 0.1 M PBS. Carefully mount slices on gelatinized slides using a soft brush, air dry overnight at room

temperature. Counterstain, add permanent mounting medium and place coverslips. Store at 4 °C for up to 6 months.

Detection of BrdU using peroxidase reaction with the avidin-biotin-peroxidase complex. Transfer slices from the cryoprotection solution to 0.1 M PBS to bring to room temperature. Rinse 3 times for 10 min each with 0.1 M PBS. Incubate for 30 min with endogenous peroxidase blocking solution to inactivate endogenous peroxidase. Rinse 3 times for 10 min each in 0.1 M PBS. Optionally, perform antigen retrieval. Incubate for 20 min with 2 N HCl at 37 °C. Rinse in 0.1 M borate buffer (pH 8.5) for 10 min. Wash 3 times for 10 min each with ice-cold 0.1 M PBS. Incubate for 2 h at room temperature in PBS++ (blocking solution). Incubate with anti-BrdU primary antibody (mouse host) 1:250 in PBS+ overnight at 4°C. On day 2, rinse 3 times for 10 min each with 0.1 M PBS. Incubate with 1:250 biotinylated secondary antibody (anti-mouse) in PBS+ for 2–4 h at room temperature. Rinse 3 times for 10 min each with 0.1 M PBS. Incubate in the ABC solution for 1 h at room temperature. Rinse 3 times for 10 min each with 0.1 M PBS. Transfer slices to DAB peroxidase (HRP) substrate solution and incubate for 2–10 min. When slices become dark grey, visualize the tissue with a magnifying glass or a microscope. If positive cells are present, rinse 3 times (15 min each) with tap water (to reduce background) followed by 3 times with 0.1 M PBS wash for 10 min each. Carefully mount slices on gelatinized slides using a soft brush and then air dry overnight at room temperature. Counterstain if needed, add permanent mounting medium and place coverslips. Store at 4 °C for up to 6 months.

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