863 The role of Notch and Ras/MAPK signaling pathways in the progression of human breast cancer

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Background: Breast cancer is a leading cause of death among women world over including India. Even though various studies have implicated aberrant Notch signaling in breast cancer, the pattern of expression of Notch receptors, ligands and target molecules or the molecular mechanism is not clearly defined. To address this, in this study we have undertaken a detailed immunohistochemistry based expression analysis of various Notch receptors, ligands and downstream targets at different stages of breast cancer progression.
Materials and Methods: A detailed immunohistochemistry analysis was performed on various breast cancer and normal samples. Different cell culture assays including soft agar colony formation assay, in vivo tumourigenicity assay, mammosphere formation assay were performed for this study.
Result: Our study shows that there is a significant increase in the expression of Notch receptor (Notch1,2,4) and ligands (Jagged1,2 and DLL4) in breast cancers compared to their normal counterparts. We detected active cleaved Notch1 and downstream targets Hes1/Hes5 in more than 75% of the breast cancer tissues analyzed. The cleaved Notch 1 and Hes1/Hes5 were found to be up-regulated as early as hyperplastic and DCIS stages of the cancers which indicated the that aberrant Notch pathway could be an early event during breast carcinogenesis. To assess the role of Notch1 in mammary epithelial cell transformation, we over-expressed constitutively active Notch1 (AcN1) into immortalized mammary epithelial cells (HMLEs). AcN1 was able to transform HMLEs only when co-expressed with low amounts of oncogenic Ras. This co-operation of AcN1 and Ras/MAPK pathway is also reflected in vivo, as a subset of cleaved Notch1 positive tumours additionally expressed phopsho-Erk1/2 in the nuclei. These cases were aggressive grade III carcinomas with high node positivity suggesting Notch-Ras co-operation could lead to poor prognosis. This suggests that combined targeting of Notch and Ras/MAPK pathway molecules could be the new modality in breast cancer treatment.
Conclusion: High level expression of Notch receptors and ligands, and its increased activation in several breast cancers and early precursors, places Notch signaling as a key player in breast cancer and its progression.

864 Evolutional epidemiology of human papillomavirus genotyping and multiplicity for the triage of Korean women with abnormal cytology by longitudinal prospective study

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Background: Despite carcinogenesis of HPV infection, the pathologic behavior of other HPV types is still unclear. With surveyed by recently available HPV DNA chip, where 22 HPV probes harbor, it has been possible to analyze evolutional course according to HPV genotypes in abnormal PAP cytology findings.
Material and Methods: 1983 patients were enrolled in the follow-up program of the cytology and follow-up HPV genotyping triage arms in Yonsei University College of Medicine and Cha Medical College Hospital up to 5 years. All patients were checked by HPVDNAChip together with PAP cytology smear at least more than twice (up to 7 times in 5 years) in subsequent 40 months. 574 patients were censored for those with HPV infections in any follow-up periods. GEE method with sequential association analysis and decision tree analyses were performed for regression analysis.
Results: Spontaneous regression from initial HPV in cases ranged from reactive condition to ASCUS or LSIL was identified in 66.72% of a total of population in the mean time of 14.8 months. With relation to cytological diagnosis, HPV persistent or progressive metatyping (changed to higher risk types; 12.72%) was significantly higher risk factor to develop HSIL or SCC than HPV regression (66.72%) or regressive metatyping (altered to lower risk types; 1.92%). HPV 16 (16.4%), 35 (68.4%), 52 (40.9%) and 58 (26.7%) were commonly associated with persistent infection. Persistent infection of HPV 16, 35 and 58 were found to be significantly higher risk factor of HSIL or SCC than that of HPV 52. Increased risk of initial HPV revealed significantly increased severity of cytological diagnosis by 1.25 in the stepwise regression analysis ($p < 0.0001$). In terms of HPV persistence, the possible rate of HSIL and SCC was 41.2% and 70.6% in persistence of intermediate risk of HPV (30 and 50 series), respectively. HSIL or SCC with HPV persistent or progressive metatyping pattern was observed as often as three or four times than those with HPV regression pattern. 14.3% of low risk HPV effect on HSIL. Age ranged from 32 and 37 yrs mostly effects on HSIL, whereas age from 37 to 48 yrs being on SCC. SCC showed HR persistence in 55.56%. Multiple infection more than two different genotypes was encompassing 7.7% of total population and 30.8% of HPV positive patients in the last examination, whereas being 17.1% and 25.9% in the initial HPV examination, respectively.
Conclusion: HPV progressive metatypings are as high persistent infections of HPV types in the risk factor of cervical lesions. HSIL and SCC were significantly prevalent in HPV persistence or progressive metatyping, whether it is single or multiple, especially in HPV 16, 35, and 58.

865 Elevated L1CAM expression mediates malignant transformation and enhances tumourigenicity of pancreatic ductal epithelial cells

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Background: Pancreatic ductal adenocarcinoma (PDAC) exhibits a strong desmoplastic reaction with stromal pancreatic myofibroblasts (PMFs), and PMFs are supposed to substantially drive PDAC tumourigenesis. Previously, we observed high expression of the adhesion molecule L1CAM in PDAC cells accounting for chemoresistance. Our present study investigates whether PMFs and L1CAM promote malignant transformation of pancreatic ductal epithelial cells and increase their tumourigenicity.
Material and Methods: Immortalized human pancreatic ductal epithelial cells (HPDE) were cocultured with freshly isolated PMFs up to 6 weeks in a transwell setting. Apoptosis of HPDE cells was measured using a caspase-3/-7 activity assay, and cell transmigration was determined using a modified Boyden chamber. L1CAM dependency was analysed by siRNA-mediated knockdown of L1CAM expression or by stable transfection with L1CAM cDNA (HPDE-L1CAM cells). Tumourigenicity of HPDE cells was proven by intrapancreatic inoculation of HPDE cells into SCID mice and follow-up examination by high-resolution ultrasound.
Results: After coculture with PMFs or TGF-b1 stimulation, HPDE cells acquire a spindle-like cell morphology along with increased expression level of the mesenchymal marker proteins vimentin and N-cadherin as well as activation of the transcription factor Slug. Furthermore, a strong TGF-b1 and Slug dependent increase of L1CAM expression was observed, accounting for elevated cell migration and chemoresistance. Knockdown of L1CAM expression reversed the chemoresistant and migratory phenotype of cocultured and TGF-b1 stimulated HPDE cells. Inoculation of HPDE cells with PMFs (HPDEco) resulted in an increased tumour burden (7/8 animals) and metastasis (skin in 6/8 and liver in 4/8 animals) compared to mice injected with HPDE cells alone (2/7 animals with tumour, no metastases). Moreover, inoculation with HPDE-L1CAM cells led to tumour growth in 5/7 animals in contrast to 1/7 animals injected with control transfected cells. Finally, treatment with L1CAM blocking antibodies clearly diminished tumour growth in mice harbouring HPDEco tumours.
Conclusion: Our data demonstrate that PMFs contribute to the malignant transformation of pancreatic ductal epithelial cells through upregulation of L1CAM, thereby elevating their tumourigenic potential. Since L1CAM seems to be essential for tumour outgrowth, cell migration and chemoresistance it represents a promising molecular target for PDAC therapy.

866 Senescent fibroblasts secretome promote tumoural initiation of normal human keratinocytes through cellular activation, enhanced migration and ECM remodelling

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Background: Carcinomas are the most frequent human cancers, and their occurrence is linked to advanced age. Using normal human epidermal keratinocytes (NHEKs), we recently showed that a fraction (10^{-2}-10^{-4}) of the NEHK population emerges from senescence in the form of neoplastic cells, re-proliferate until a second growth plateau from which a second similar emergence may occur. Emergent cells induce skin hyperplasia and carcinoma in vivo [Cancer Res, 2009. 69, 7917−25]. There is a growing interest in the role of the ageing microenvironment in the cancer development. We studied here the contribution of normal human dermal senescing fibroblasts to the initiation of neoplasic emergence from NHEK cultures.

Material and Methods: We have compared the secretomes from senescent versus young fibroblasts using secretome proteomics, combined with Western-blotting, ELISA, RT-PCR and zymographies. A fraction of NHEK growth culture medium was replaced by fibroblast (either young or senescent)-conditioned medium to investigate the secretome impact on neoplastic initiation of NHEKs.
Results: Four major groups of proteins were modulated in senescent vs young fibroblast secretomes. The expressions of extracellular matrix collagens, SPARC, and decorin were strongly affected in senescent fibroblasts secretome; expression and activation of numerous metalloproteinases were promoted, while the expression of their inhibitors was reduced; growth factors (among which HGF/SF) and cytokines were overexpressed in association with the loss of anti-angiogenic molecules. Senescent fibroblasts could then relevantly contribute to a tumour-promoting environment. Hence, the replacement of a fraction of primary NEHKs' growth culture medium by conditioned medium from normal primary senescent fibroblasts induced a strong promotion of the neoplastic initiation from primary NHEKs emerging from senescence in our culture model. It led to the acquisition of enhanced migratory and scattering capacities, and the development of small clones in soft Agar.
Conclusions: These results point to the microenvironment of normal aging fibroblasts as a factor promoting initial changes in normal human keratinocytes emerging from replicative senescence in vitro that result in cancerous phenotype.

867 Long-term GLI1 expression induces mammary gland tumour formation in nulliparous transgenic mice

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Background: The main effectors of the Hedgehog (Hh) signalling pathway are the zinc finger transcription factors of the GLI family. In human breast cancer, up regulation of GLI1 expression correlates with unfavourable overall survival. We have previously shown that multiparous conditional transgenic mice expressing GLI1 develop hyperplastic lesions and tumours. Furthermore, the Hh pathway is thought to be involved in the regulation and maintenance of CD44 positive breast cancer stem cells. Skin stem cells with active Hh signalling pathway as well as intestinal stem cells express the orphan G protein coupled receptor LGR5. The expression pattern and role of LGR5 in mammary gland tissue and cancer is not known.
Material and Methods: GLI1 expression was induced, up to 108 weeks, in female transgenic mice (MMTVrtTA;TREGLI1 and MMTVrtTA;TREGLI1;Lgr5-LacZ). The mice were monitored for the occurrence of tumours. Palpable tumours and hyperplastic lesions developed in the mice with induced GLI1 expression. Normal and tumour tissue were analysed by immunohistochemistry.
Results: Hyperplastic lesions and palpable mammary gland tumours, including solid and acinar adenocarcinomas, developed in the nulliparous mice after long-term low level GLI1 expression. Both cytokeratin 5 (K5) and cytokeratin 6 (K6) positive tumour cells were detected. Only few tumours also harboured some cytokeratin 18 (K18) positive cells. The expression of the stem cell marker CD44 was increased in the mammary ducts and tumours in the GLI1 positive mice. Lgr5 was expressed in the basal cell layer of the large mammary ducts as well as in the GLI1 induced tumours.
Conclusions: Mammary gland specific, long-term expression of GLI1 induces formation of different types of K5 and K6 positive tumours with basal character in transgenic mice. Induction of various types of tumours and expression of Lgr5 in the tumours as well as increased expression of the stem cell marker CD44 indicate that the expression of GLI1 affects mammary stem cells.

868 Genotoxicity/clastogenicity of ptaquiloside, the bracken (Pteridium aquilinum) carcinogen, towards human peripheral blood lymphocytes

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Background: Ptaquiloside (PTA), a bracken toxin, is a known carcinogen for animals but its implications on public health remain controversial (Yamada *et al*., 2007). This work addresses PTA's genotoxicity for human peripheral blood lymphocytes.
Material and Methods: PTA was isolated from bracken shoots collected in Ponte da Barca, Portugal, following methods by Ojika *et al*. (1985). Nuclear magnetic resonance techniques were used to confirm the compound's identity. The alkaline comet assay was performed according to Costa *et al.* (2008) on cells from 10 healthy donors exposed to 5 μg/ml PTA (or DMSO, negative control) in RPMI at 37ºC, for 5, 10, 20, 30, 40 or 50 min. Electrophoresis took place at 30 V for 20 min. Comet Assay IV (Perspective Instruments) software was used for slide analysis and for calculating tail intensity (TI). For chromosomal aberrations (CA) (5 donors) and sister-chromatid exchanges (SCE) tests (2 donors) cells were cultivated on supplemented RPMI. PTA was added at 24h (5, 10 or 20 μg/ml final dilutions). Bromodeoxyuridine was added to replicate cultures for SCE. Cells were then incubated for 48h and harvested after colcemid arrest. CA and SCE were counted on 100 and on 25 metaphases for each donor, respectively.
Results: The TI values for control/exposed cells at 5, 10, 20, 30, 40 and 50 min were 4.85/6.17, 5.11/5.75, 3.61/22.60, 4.77/28.53, 1.76/12.76, 1.62/10.52, respectively. Cytogenetic results were expressed, for controls and for each PTA dilution (5, 10 or 20 μg/ml) as the mean percentage of aneuploid cells (3, 15.3, 22.7, 46.4 respectively) and cells with chromosome/chromatid gaps and breaks (0.2, 2.4, 7.2, 14.5), mean number of gaps and breaks per 100 cells (0.2, 2.4, 7.8, 16.4), and the mean number of SCE per cell (9.4, 14.2, 18.4, 25.7).
Conclusions: The comet assay demonstrated that even PTA doses as low as 5 μg/ml are enough to induce DNA damage in a human *in vitro* model. Maximum damage was observed at 20−30 min, diminishing at 40−50 min, presumably due to DNA repair mechanisms. The cytogenetic tests show that at 48 h, despite such mechanisms, PTA originates structural and numeric CA and increased SCE in a dose-dependent manner. This suggests that PTA exerts its genotoxicity through multiple mechanisms and further support the hypothesis that PTA represents a significant threat to public health.

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869 The lichen compound usnic acid disturbs mitochondrial function and induces autophagy in cancer cells

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Background: The lichen compound usnic acid (UA) is a component of the fat-burner Lipokinetix and has been shown to reduce ATP production in liver cell mitochondria. The effect on mitochondria can be directly related to the property of UA to shuttle protons across membranes. Autophagy is a process that can aid cell survival during nutrient shortage. UA inhibits the growth and proliferation of cancer cells but does not induce apoptosis.
Aims and Methods: To test for changes in inner membrane mitochondrial potential using JC-1 staining and measure levels of cellular ATP in UA-treated breast and pancreatic cancer cells. Also, to test if cells treated with UA showed signs of autophagy, using electron microscopy and immunostaining for the autophagosomal marker LC-3 and Western blotting for the autophagosomal cargo p62.
Results: A drop in inner membrane mitochondrial potential was demonstrated and reduced levels of ATP were observed in breast and pancreatic cancer cells treated with 5 μg/mL and 10 μg/mL of UA for 24 hours. Clear signs of autophagy were seen after treatment with UA, but results indicate that degradation of p62 does not occur. Therefore, in ongoing experiments we are testing for autophagosomal-lysosomal fusion and acidification using a tandem-tagged mRFP-GFP-LC3 fusion construct.
Conclusion: UA treatment of cancer cells causes a drop in mitochondrial membrane potential leading to reduced ATP production. This stimulates autophagy but apparently without degradation of autophagosomal content.

870 The lichen compound protolichesterinic acid affects lipid metabolism and induces ER stress in cancer cells

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Background: The lichen metabolite protolichesterinic acid (PA) is a potent inhibitor of 5- and 12-lipoxygenase and has anti-proliferative effects on several types of cancer cells, as well as inducing apoptosis in multiple myeloma cells. Fatty acid synthase (FAS) is highly expressed in human carcinomas and appears to be required for their survival. The chemical structure of PA is very similar to known FAS inhibitors. Aims and methods: To test if PA inhibited FAS by measuring uptake of ^{14}C-acetate into cells and to test for ER-stress, which is a known consequence of FAS inhibition, using Western blotting for phosphorylated-eIF2α. Signalling through major stimulatory pathways was tested by measuring activation of ERK1/2 and STAT3.
Results: Uptake of ^{14}C-acetate into breast cancer cells was reduced in a dose-depedent manner by PA reaching 33% at 10 μg/mL. The same concentration