Metabolic profiling of changes in the transition from Pre-invasive to Invasive Cervical Cancer using Magic Angle Spinning Magnetic Resonance Spectroscopy of intact tissues

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Introduction: The development of invasive cervical cancer is preceded by a well-defined pre-invasive stage, cervical intraepithelial neoplasia (CIN). Elevated levels of choline have previously been identified in a number of tumours including cervical cancer [1] but whether these metabolites are elevated in CIN tissue is still unclear. The aim of this study, therefore, is to investigate the metabolic changes in the transition from normal-to-pre-invasive, and from pre-invasive to invasive cervical cancer using ^{1}H and ^{31}P HR-MAS MRS of intact tissues in which the tissue content was subsequently confirmed with histology

Methods: **Tissue Collection** Women were studied with written informed consent and local ethics committee approval. Targeted biopsies were obtained on colposcopy in cervical intraepithelial neoplasia, CIN, (low-grade n=5, high-grade n=40) and on visual inspection when invasive disease was clinically obvious (n=23). Cervical tissues were also obtained from women with normal smears undergoing hysterectomy for benign uterine disease (n=5). Tissue samples were frozen within 5 mins of excision and stored at –80ºC prior to MR analysis.

Ex vivo MAS studies Magic Angle Spinning (MAS) Magnetic Resonance ^{1}H spectra were acquired on a Bruker Avance 11.74T spectrometer (spin rate 3kHz, temp 4°C, CPMG sequence TR=4.8s, 512 scans, 41min acquisition, internal reference creatine 3.027 ppm). ^{31}P MAS spectra were obtained using a 1H decoupled pulse-acquire sequence (TR= 2.82s, 2048 scans, 96min acquisition, external reference methylene diphosphonic acid). Peak assignments were based on chemical shift. Peak areas were measured using the AMARES algorithm [2].

Histopathology Following MAS, tissue samples were fixed in formalin, embedded in paraffin, routinely processed and stained with H&E. Sections were reviewed by a pathologist to determine if samples were primarily normal, CIN or tumour.

Analysis Statistical analysis of the data was performed using SPSS, version 14 for Windows XP. Normality plots showed that metabolite data were not normally distributed; the natural log of values was therefore used to normalize the data. Metabolite concentrations from 1H and 31P spectra were initially evaluated based on patient category at diagnosis. These groups were further divided and compared based on histological classification of tissue samples. One-way analysis of variance (ANOVA) with Bonferroni correction was used to investigate overall differences between groups. A p-value of less than 0.05 was chosen as a criterion for statistical significance. Independent two-sample t-tests adjusted for multiple comparisons were used to identify significant differences between individual groups.

Results and Discussion

Smear result	Aln (^{1}H)	Cr (^{1}H)	Cho (^{1}H)	PC (^{1}H)	Tau (^{1}H)	PE (^{31}P)	PC (^{31}P)	Pi (^{31}P)
No Dyskaryosis (normal cervix) N=5	0.64± 0.18	1.02± 0.18	0.60± 0.33	0.91± 0.30	1.34± 0.32	1.34± 0.28	0.63± 0.66	4.07± 1.01
Mild Dyskaryosis N=5	0.68± 0.15	1.02± 0.39	0.57± 0.44	1.05 ±0.41	1.02± 0.30	NA	NA	NA
Moderate/Severe Dyskaryosis N=40	0.64± 0.40	1.02± 0.69	0.49± 0.28	1.08± 0.54	0.98± 0.63	NA	NA	NA
Cancer N=23	0.76± 0.58	0.75± 0.47	0.90± 0.54	2.08± 01.46	1.02± 0.52	5.61± 2.89	2.12± 1.60	9.70±4. 6
p (ANOVA)	**0.92**	**0.13**	**0.004**	**0.01**	**0.48**	**0.0001**	**0.65**	**0.004**

Table 1 Comparison of metabolite concentrations (µmol/g wet wt) (mean ± sd) in normal, dyskaryotic and cancer tissue based on diagnostic smear test. NA- not analysed.

Initial smear result	group	Histology of biopsy studied by MRS	Aln (^{1}H)	Cr (^{1}H)	Cho (^{1}H)	PC (^{1}H)	Tau (^{1}H)	PE (^{31}P)	PC (^{31}P)	Pi (^{31}P)
No Dyskaryosis	1	**Normal N=5**	0.64± 0.18	1.02± 0.18	0.60± 0.33	0.91± 0.30	1.34± 0.32	1.34± 0.28	0.63± 0.66	4.07± 1.01
Dyskaryosis Present	2	**Normal N=18**	0.65± 0.34	0.97± 0.62	0.50± 0.25	1.11± 0.56	1.21± 0.75	NA	NA	NA
	3	**Low grade CIN N=15**	0.61± 0.35	1.07± 0.61	0.58± 0.39	1.03± 0.45	0.84± 0.48	NA	NA	NA
	4	**High grade CIN N=12**	0.68± 0.48	1.02± 0.82	0.39± 0.13	1.06± 0.60	0.83± 0.39	NA	NA	NA
Cancer	5	**Normal N=8**	0.40± 0.18	0.44± 0.29	0.62± 0.30	1.35± 1.26	0.85± 0.53	4.96± 2.10	1.70± 1.75	8.73± 4.81
	6	**CIN N=3**	0.70± 0.09	0.97± 0.09	1.16± 0.44	1.55± 0.85	0.79± 0.61	3.83± 1.12	1.62± 0.69	11.31± 2.48
	7	**Cancer N=12**	1.12± 0.64	0.90± 0.52	1.00± 0.63	2.71± 1.48	1.21± 0.54	6.76± 3.18	2.55± 1.70	9.66± 5.11
p (ANOVA)			**0.03**	**0.01**	**0.009**	**0.003**	**0.24**	**0.0001**	**0.93**	**0.13**

Table 1 Metabolite concentrations (µmol/g wet wt) (mean ± sd) obtained from patients with diagnostic biopsies positive for CIN or cancer based on the histological analysis. NA-not analysed

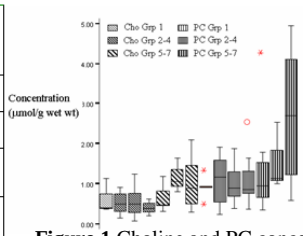

Figure 1 Choline and PC concentrations (^{1}H NMR) in different tissue groups (lower quartile, bottom line of the box; median, middle line of the box; upper quartile, top line of the box; lower whisker, lower value; upper whisker, upper value), * extreme values and o outliers

For tissue types grouped by patient category based on smear result, the overall group differences were significant for mean tissue concentrations of Cho and PC. Independent two-sample t-test showed that the mean tissue concentrations of Cho and PC were significantly increased in cancer patients compared to patients with severe dyskaryosis. Differences between non-cancer groups were not significant. PE and Pi mean tissue concentrations were significantly higher in tissue from cancer patients compared to tissue from normal patients. When samples were further subdivided by histopathology, mean tissue concentrations of Aln, Cr, Cho and PC between the groups were found to be statistically significant. Independent samples t-tests showed concentrations of Cho and PC[^{1}H] to be significantly increased in cancer compared to high-grade CIN from patients with dyskaryosis. PE concentration was significantly increased between normal tissue from patients with histologically normal cervix and cancer. Significant reductions in mean tissue concentrations of Aln and Cr were observed in histologically normal tissue from cancer patients compared to normal tissue from non-cancer patients. There was a significant increase in mean concentration of Cho in CIN tissue from cancer patients compared to high-grade CIN tissue from patients with dyskaryosis. It is possible that in our study increased choline transport is seen in these virally transformed CIN cells at a point where disruption of the cell cycle has occurred and malignant transformation is occurring. There also may be an increased rate of extracellular choline uptake in abnormal cells close to a malignancy. The increase in choline containing metabolites in CIN tissue from cancer patients could be due to microscopic foci of cancer cells contaminating the sample. Alternatively, it is possible that the type of CIN that progresses to cancer is metabolically different from CIN that does not show this progression. The reduction in metabolites such as alanine and creatine in normal tissue from cancer patients compared to normal tissue from non-cancer groups may be explained by metabolite depletion due to increased metabolism in the neighboring cancer. It is likely that the presence of tumour alters the metabolic environment of the surrounding normal tissue

Conclusion HR-MAS spectral analysis shows significant differences between invasive cervical cancer and high-grade CIN. Increased levels of choline-containing compounds are observed in cancer tissue. Our findings also suggest that normal tissue adjacent to tumour shows some metabolite depletion, while concentration of choline-containing metabolites is increased in CIN tissue adjacent to a malignancy. Changes in choline metabolism may be a useful biomarker in identifying the transition of pre-invasive to invasive disease.

References [1] deSouza, NM *et al*. NMR in Biomedicine, 2004;17:144-153. [2] L Vanhamme JMR **129**:

Acknowledgement: *This work was supported by Cancer Research UK [CUK] grant numbers C1060/A5117 and C1353/A5408)*