Notch-induced transcription factors are predictive of survival and 5-fluorouracil response in colorectal cancer patients

P A Candy¹,², M R Phillips², A D Redfern¹,²,³, S M Colley¹,², J A Davidson²,³, L M Stuart¹,², B A Wood⁴, N Zeps⁵ and P J Leedman*¹,²

¹Laboratory for Cancer Medicine, University of Western Australia Centre for Medical Research, Western Australian Institute for Medical Research (WAIMR), Perth, Western Australia 6000, Australia; ²School of Medicine and Pharmacology, University of Western Australia, Perth, Western Australia 6009, Australia; ³Medical Oncology, Royal Perth Hospital, Perth, Western Australia 6000, Australia; ⁴PathWest Laboratory Medicine WA, QEII Medical Centre, Perth, Western Australia 6009, Australia and ⁵Bendat Family Comprehensive Cancer Centre, St John of God Hospital, Subiaco, Western Australia 6008, Australia

Background: The purpose of this study was to evaluate the expression of Notch-induced transcription factors (NTFs) HEY1, HES1 and SOX9 in colorectal cancer (CRC) patients to determine their clinicopathologic and prognostic significance.

Methods: Levels of HEY1, HES1 and SOX9 protein were measured by immunohistochemistry in a nonmalignant and malignant tissue microarray of 441 CRC patients, and the findings correlated with pathologic, molecular and clinical variables.

Results: The NTFs HEY1, HES1 and SOX9 were overexpressed in tumours relative to colonic mucosa (OR = 3.44, P < 0.0001; OR = 7.40, P < 0.0001; OR = 4.08 P < 0.0001, respectively). HEY1 overexpression was a negative prognostic factor for all CRC patients (HR = 1.29, P = 0.023) and strongly correlated with perineural and vascular invasion and lymph node (LN) metastasis. In 5-fluorouracil (5-FU)-treated patients, the tumour overexpression of SOX9 correlated with markedly poorer survival (HR = 8.72, P = 0.034), but had no predictive effect in untreated patients (HR = 0.70, P = 0.29). When HEY1, HES1 and SOX9 expression were combined to predict survival with chemotherapy, in treated patients there was an additive increase in the risk of death with each NTF overexpressed (HR = 2.09, P = 0.01), but no prognostic import in the untreated patient group (HR = 0.74, P = 0.19).

Conclusion: The present study is the first to discover that HEY1 overexpression correlates with poorer outcome in CRC, and NTF expression is predictive of CRC patient survival with 5-FU chemotherapy. If confirmed in future studies, testing of NTF expression has the potential to enter routine pathological practice for the selection of patients to undergo chemotherapy alone or in combination with Notch inhibitors.

Colorectal cancer (CRC) is the third most common malignancy and the second highest cause of cancer death in USA (Jemal et al, 2011). Colorectal cancer may be categorised on the basis of its site of emergence into proximal colonic, distal colonic or rectal carcinoma, each with distinct demographic, risk factors, molecular and pathological markers, and clinical outcomes (Li and Lai, 2009). Aberrant Notch signalling has been implicated in chemotherapy treatment resistance (Schreck et al, 2010; Miyamoto and Rosenberg, 2011; Izrailit and Reedijk, 2012). In the malignant state, Notch signalling promotes epithelial-to-mesenchymal transition (EMT), chemoresistance, proliferation and metastasis, and activates key pathways, such as cyclooxygenase 2 (COX2), NF-κB and Wnt signalling (Meng et al, 2009; Xie et al, 2011; Zhang et al, 2011; Garcia and Kandel, 2012). Numerous approaches for silencing Notch signalling have been explored, with gamma-secretase inhibitors (GSIs) and siRNAs showing the most promise.
(Purow et al., 2005; Fan et al., 2010). Many new specific Notch inhibitors are under development, with 23 ongoing phase I/II clinical trials investigating the efficacy of GSIs (www.clinicaltrials.gov). However, enthusiasm regarding the successes with in vitro and xenograft models has been tempered by the lack of efficacy as single-agent therapy in CRC as well as significant mucosal toxicity (Staal and Langerak, 2008; Strosberg et al., 2012). Treatment in concert with chemotherapy shows promise in the preclinical setting, but remains unexplored in patients. Thus, there is a need to better understand the intricacies of Notch signalling effects to better guide the future development and application of Notch inhibitors.

If predictive or prognostic factors do exist within this pathway, the downstream effectors of Notch activation, the Notch-induced transcription factors (NTFs), in particular HEY1, HES1 and SOX9, are appropriate candidates for further study and the focus of this investigation. Upon Notch activation, there is an increase in the expression of HEY1, HES1 and SOX9, which consequently accumulate in the nucleus, leading to the transcriptional regulation of downstream targets (Maier and Gessler, 2000; Kunnimalaiyaan et al., 2005; Muto et al., 2009). HEY1 has roles in cardiac embryology, osteogenesis and neurogenesis (Sakamoto et al., 2003; Zamurovic et al., 2004; Belandia et al., 2005; Fischer et al., 2007). It is a mediator of Notch signalling in cancer, through regulation of differentiation, tumour suppressor P53, androgen receptor signalling and TGF-β-dependent EMT (Yang and Weinberg, 2008; Villaronga et al., 2009). In neural stem cells, HEY1 promotes proliferation. Furthermore, overexpression in glioblastomas and pancreatic cancer correlated with higher tumour grade and poorer patient survival (Hulleman et al., 2009; Mann et al., 2012). To date, HEY1 tumour expression has not been studied in CRC patients. HES1 acts either as an oncogene or as a tumour suppressor, depending on the tumour context. Implicated mechanisms include regulation of proliferation, EMT, metastasis and chemoresistance (Hughes, 2009; Meng et al., 2009; Kannan et al., 2011; Lage et al., 2011; Ueo et al., 2012). Clinical studies of HES1 overexpression in cancer have shown poorer prognosis in ovarian malignancy, either no impact or a modest trend towards poorer overall survival in two small CRC cohorts and no prognostic association in biliary tract cancer (Reedijk et al., 2008; Wang et al., 2010; Mazur et al., 2012). SOX9 directly promotes HES1 expression, has a critical role in embryogenesis and is required for development, differentiation and lineage commitment in numerous tissues including the intestinal epithelium (Müller et al., 2010; Mathieu et al., 2012). In cancer, SOX9 regulates proliferation, differentiation and metastasis (Bastide et al., 2007; Mathieu et al., 2012). Clinical studies in breast, gastric biliary tract and CRC indicate poorer prognosis with tumour SOX9 overexpression (Lü et al., 2008; Zhou et al., 2011; Mathieu et al., 2012; Mazur et al., 2012). Hence, considering the mechanistic evidence for the roles of HEY1, HES1 and SOX9 in cancer pathogenesis and the preclinical data implicating Notch signalling chemotherapies, we set out to characterise the expression of these NTFs in CRC and to explore a potential role in chemotherapies resistance. To this end, this study seeks to determine the prognostic, predictive and clinicopathologic significance of these NTFs and their consequent suitability as biomarkers in determining which patients will benefit most from Notch inhibitor therapy using a CRC tissue microarray (TMA).

### Materials and Methods

**Study populations.** Colorectal tissues were collected between 1990 and 1999 from 1056 consenting CRC patients undergoing surgery at Sir Charles Gairdner Hospital (SCGH), Western Australia. Information on patient demographics and tumour features were obtained from SCGH medical records and previous studies of this cohort (Chai et al., 2004; Feng Han et al., 2006; Salama et al., 2009). Tumour site was classified as ‘rectal’ when in the rectum, ‘distal’ when occurring between the proximal rectal margin and the splenic flexure and ‘proximal’ when proximal to and within the splenic flexure. Samples from a total of 280 patients with AJCC stage II (48% male, 52% female) and 161 patients with stage III CRC (49% male, 51% female) were stained for HEY1, HES1 and SOX9 protein expression (n = 441). Information on tumour stage, anatomic site, histologic grade, vascular invasion, perineural invasion, 5-flouracil (5-FU) chemotherapy, microsatellite instability (MSI) determined by BAT-26 marker, β-catenin, COX2, P53, MLH1 and SFRP4 was available. Information on disease-specific survival was obtained from the Cancer Registry of Western Australia. The great majority of cases received 5-FU chemotherapy including folinic acid for 6 cycles, with a minority of older cases receiving 5-FU chemotherapy including levamisole for 12 cycles, each of 4 weeks duration. All rectal cancer patients were treated prior to the introduction of neoadjuvant therapy as the standard of care for local advanced disease. The median follow-up time was 69.7 months for patients with AJCC stage II disease and 52.4 months for patients with stage III disease. At the end of the study period, 31% of patients had died as a result of disease recurrence and 25% had died from unrelated causes. Ethics approval for this study was obtained from the Royal Perth Hospital Human Research Ethics Committee.

**Tissue microarray.** The TMA was constructed as described previously (Chai et al., 2004). The array comprised 1056 surgically resected cases of which 441 cases were examined for NTF expression in this study. For each case, three cores of 1-mm diameter were taken at random from tumour tissues, and an additional two 1-mm diameter cores were taken from histologically normal colonic mucosa.

**Immunohistochemistry.** Sections were deparaffinized through three changes of xylene (3 min), rehydrated through graded alcohols to distilled water and subjected to antigen retrieval in EDTA (pH 8.0) under pressure. After blocking endogenous peroxidase activity with hydrogen peroxide (HRP), either SOX9 (SC-20095, Santa Cruz, Dallas, TX, USA; 1:75), HES1 (AB5702, Millipore, Billerica, MA, USA; 1:3000) or HEY1 (AB2614, Abcam, Cambridge, MA, USA; 1:75) antibody was applied for 60 min. Dako Envision + Dual link System–HRP was then applied for 30 min incubation. Sections were visualised with diaminobenzidine (Dako, Campbellfield, VIC, Australia) followed by a light counterstain of haematoxylin.

**TMA scoring.** Examination of HEY1, HES1 and SOX9 expressions were blinded to patient clinical data and overseen by a pathologist. At least 150 tumour cells or colonocytes were evaluated per case. Expression was classified based on epithelial cell staining intensity and percent positivity and represented as scores of 0 (absent), 1 (weak), 2 (moderate) and 3 (strong) (Figure 1). For comparison with clinical variables and survival, the full semi-ordinal expression levels of HEY1, HES1 and SOX9 ranging 0–3 in 0.25 increments were used. Dichotomised ‘high’ and ‘low’ HEY1, HES1 and SOX9 immunoreactivities were used in survival curves and comparisons of staining densities across tissues by classifying as high, tissue sections that had moderate or strong staining in ≥50% of epithelial cells, with the remainder classified as low.

**Data analysis.** Spearman’s ρ was used for the determination of bivariate associations between ordinal NTF expressions and clinical variables. Kaplan–Meier survival curves were produced using the R ‘survival’ package. Associations with survival were examined with Cox’s proportional hazards models, with ties addressed by Efron’s
Overexpression strongly correlated with higher stage (malignant tissue and HEY1 (r = 0.382, P < 0.005) and SOX9 (r = 0.190, P < 0.005) in malignant tissue and HEY1 (r = 0.241, P < 0.005) and SOX9 (r = 0.149, P < 0.05) in nonmalignant tissue. In addition, HEY1 overexpression strongly correlated with higher stage (r = 0.221, P < 0.005), LN metastasis (r = 0.255, P < 0.005), overexpression of the proinflammatory prostaglandin synthase COX2 (r = 0.287, P < 0.005), DNA mismatch repair protein MLH1 (r = 0.136, P < 0.05), the apoptotic inducer SFRP4 (r = 0.102, P < 0.05), the CRC oncogene β-catenin (r = 0.166, P < 0.005), vascular (r = 0.134, P < 0.05) and perineural (r = 0.160, P < 0.05) invasion, and inversely correlated with MSI (r = −0.106, P < 0.05). SOX9 overexpression in tumours correlated with vascular invasion (r = 0.123, P < 0.05), and high expression of HES1 (r = 0.190, P < 0.005) and SOX9 (r = 0.190, P < 0.005) in malignant tissue and SOX9 (r = 0.304, P < 0.005) in nonmalignant tissue. Elevated HES1 expression in tumours correlated with overexpression of the critical tumour suppressor P53 (r = 0.101, P < 0.05), HEY1 (r = 0.382, P < 0.005), SOX9 (r = 0.190, P < 0.005), SFRP4 (r = 0.128, P < 0.05) and β-catenin (r = 0.238, P < 0.005) in malignant tissue, and HEY1 (r = 0.235, P < 0.005) and HES1 (r = 0.130, P < 0.05) in nonmalignant tissue (Table 2). Overexpression of NTFS was not associated with tumour site, patient age or tumour differentiation.

Survival analyses. Cox regression of clinicopathologic and molecular features stratified by tumour site is shown in Table 3. Across all patients, tumour overexpression of HEY1 (HR = 1.29, P = 0.023) or COX2 (HR = 2.06, P = 0.001) correlated with poorer prognosis. HEY1 overexpression significantly predicted poorer survival in colon (HR = 1.31, P = 0.037), but not in rectal cancer.

Table 1. Comparison of the mean densities of HEY1, HES1 and SOX9 in normal colorectal mucosa and colorectal cancer tissues

| Marker | Normal | Tumour |
|--------|--------|--------|
| HEY1   | 304    | 108    | 395 | 368 | 3.44 | < 0.0001 |
| SOX9   | 301    | 89     | 392 | 363 | 4.08 | < 0.0001 |
| HES1   | 300    | 55     | 392 | 407 | 7.40 | < 0.0001 |

Abbreviations: n = number of patients; T/N = tumour/normal ratio. Mean density of the Notch transcription factors HEY1, SOX9 and HES1 in normal colonic mucosa and colorectal cancer tissues.

RESULTS

NTF protein expression. The normal and malignant colorectal tissues from 280 AJCC stage II and 161 AJCC stage III CRC patients were analysed for HEY1, HES1 and SOX9 protein expression. Distributions of protein expression are shown in Figure 1. In normal colonic epithelium, HEY1 and HES1 were highly expressed towards the bowel luminal surface, with expression reducing to none towards the base of the crypt. Conversely, SOX9 was predominantly expressed in basal crypt cells with weaker expression evident towards the bowel luminal surface. SOX9 expression was predominantly nuclear with weak cytoplasmic staining in malignant and nonmalignant tissue. Expression of HEY1, HES1 and SOX9 were predominantly expressed in the cytoplasm of patients. Expression data for each NTF had a log-normal distribution by Shapiro–Wilks tests. The expressions of HEY1, HES1 and SOX9 were significantly elevated in malignant relative to normal tissues (OR = 3.44, P < 0.0001; OR = 7.40, P < 0.0001, 4.08 P < 0.0001, respectively) (Table 1). The total percentage of patients with tumours classed as having overexpressed HEY1, HES1 and SOX9 was 61%, 68% and 60%, respectively.

NTF associations. Correlations between NTFS and clinicopathologic and molecular features are listed in Table 2. Notch-induced transcription factors showed significant coexpression across the CRC patients’ malignant and nonmalignant tissues. High HEY1 tumour expression correlated significantly with elevated levels of HES1 (r = 0.382, P < 0.005) and SOX9 (r = 0.190, P < 0.005) in malignant tissue and HEY1 (r = 0.241, P < 0.005) and SOX9 (r = 0.149, P < 0.05) in nonmalignant tissue. In addition, HEY1 overexpression strongly correlated with higher stage (r = 0.221, P < 0.005), LN metastasis (r = 0.255, P < 0.005), overexpression of the proinflammatory prostaglandin synthase COX2 (r = 0.287, P < 0.005), DNA mismatch repair protein MLH1 (r = 0.136, P < 0.05), the apoptotic inducer SFRP4 (r = 0.102, P < 0.05), the CRC oncogene β-catenin (r = 0.166, P < 0.005), vascular (r = 0.134, P < 0.05) and perineural (r = 0.160, P < 0.05) invasion, and inversely correlated with MSI (r = −0.106, P < 0.05). SOX9 overexpression in tumours correlated with vascular invasion (r = 0.123, P < 0.05), and high expression of HES1 (r = 0.190, P < 0.005) and SOX9 (r = 0.190, P < 0.005) in malignant tissue and SOX9 (r = 0.304, P < 0.005) in nonmalignant tissue. Elevated HES1 expression in tumours correlated with overexpression of the critical tumour suppressor P53 (r = 0.101, P < 0.05), HEY1 (r = 0.382, P < 0.005), SOX9 (r = 0.190, P < 0.005), SFRP4 (r = 0.128, P < 0.05) and β-catenin (r = 0.238, P < 0.005) in malignant tissue, and HEY1 (r = 0.235, P < 0.005) and HES1 (r = 0.130, P < 0.05) in nonmalignant tissue (Table 2). Overexpression of NTFS was not associated with tumour site, patient age or tumour differentiation.

Survival analyses. Cox regression of clinicopathologic and molecular features stratified by tumour site is shown in Table 3. Across all patients, tumour overexpression of HEY1 (HR = 1.29, P = 0.023) or COX2 (HR = 2.06, P = 0.001) correlated with poorer prognosis. HEY1 overexpression significantly predicted poorer survival in colon (HR = 1.31, P = 0.037), but not in rectal cancer.
patients (HR = 1.07, \( P = 0.664 \)). In contrast, elevated HES1 and SOX9 expression were not significantly associated with overall survival (HR = 1.05, \( P = 0.596 \); HR = 1.14, \( P = 0.195 \), respectively). High \( \beta \)-catenin predicted better survival in colon, but not in rectal cancer patients, which is consistent with previous studies (Gunther et al., 1998; Morikawa et al., 2011). High MSI was predictive of improved survival in patients with colon, but not rectal tumours, likely due to the very low incidence of high MSI in rectal cancer, as previously observed (Minoo et al., 2010; Table 3).

Patient outcomes were next stratified by AJCC stage and related to HEY1, HES1 and SOX9 protein expression (Table 4). Over-expression of NTF HEY1, HES1 or SOX9 was predictive of reduced survival in stage III (HR = 1.34, \( P = 0.007 \); HR = 1.23, \( P = 0.037 \); HR = 1.29, \( P = 0.022 \), respectively), but not stage II CRC cases (HR = 1.05, \( P = 0.73 \); HR = 0.98, \( P = 0.82 \); HR = 1.02, \( P = 0.83 \), respectively).

A major factor that affects survival and its prediction by NTF tumour expression, in stage III, but not stage II CRC, is the effectiveness of adjuvant chemotherapy. Consistent with clinical practice at the time, a proportion of stage III CRC cases received chemotherapy, but stage II CRC cases did not. As activation of Notch signalling has been associated with chemotherapy resistance (Schreck et al., 2010; Miyamoto and Rosenberg, 2011; Izrailit and Reedijk, 2012), we investigated whether tumour overexpression of NTFs predicted reduced survival in chemotherapy-treated patients. As shown in Figure 2, stage III CRC patients with tumour overexpression of SOX9 had poorer survival with 5-FU treatment, relative to those with low SOX9 (HR = 0.72, \( P = 0.034 \)) over a 10-year period. This was accentuated in patients with rectal cancer (\( P = 0.007 \)) (Supplementary Figure S1). A similar trend towards reduced survival was observed for patients with tumour overexpression of HEY1 or HES1 treated with chemotherapy, but this was not significant (HR = 2.07, \( P = 0.14 \); HR = 2.07, \( P = 0.14 \), respectively). In untreated patients, tumour overexpression of HEY1, HES1 or SOX9 did not correlate with poorer prognosis (HR = 0.18, \( P = 0.79 \); HR = 0.66, \( P = 0.16 \); HR = 0.70, \( P = 0.29 \), respectively) (Figure 2).

The prognoses of chemotherapy-treated and untreated stage III CRC patients were then predicted using the collective

***Table 2. Bivariate associations between elevated HEY1, HES1 and SOX9 expression and clinical variables***

| Variable                  | High tumour HEY1 | High tumour HES1 | High tumour SOX9 |
|---------------------------|------------------|------------------|------------------|
| High tumour HEY1          | \( \mu \)         | 0.190*           | 0.382*           |
| High tumour HES1          | \( \mu \)         | 0.382*           | 0.190*           |
| High tumour SOX9          | \( \mu \)         | 0.378            | 0.389            |
| High normal HEY1          | \( \mu \)         | 0.141*           | 0.106            |
| High normal HES1          | \( \mu \)         | 0.149*           | 0.104            |
| High normal SOX9          | \( \mu \)         | 0.147            | 0.104            |
| AJCC stage III            | \( \mu \)         | 0.134*           | 0.008            |
| LN metastasis*            | \( \mu \)         | 0.133            | 0.008            |
| Vascular invasion         | \( \mu \)         | 0.123*           | 0.056            |
| Perineural invasion       | \( \mu \)         | 0.001            | 0.069            |
| \( \beta \)-catenin       | \( \mu \)         | 0.021            | 0.123            |
| High COX2                 | \( \mu \)         | 0.014            | 0.035            |
| High PS3                  | \( \mu \)         | 0.030            | 0.101            |
| High MSI                  | \( \mu \)         | –0.106           | –0.071           |
| High MLH1                 | \( \mu \)         | 0.136            | 0.058            |
| High SFRP4                | \( \mu \)         | 0.102            | 0.043            |
| \( n \)                   | 265              | 264              | 264              |
| \( n \)                   | 242              | 243              | 245              |
| \( n \)                   | 377              | 370              | 374              |
| \( n \)                   | 379              | 371              | 374              |
| \( n \)                   | 383              | 376              | 380              |
| \( n \)                   | 352              | 345              | 348              |
| \( n \)                   | 381              | 373              | 378              |
| \( n \)                   | 385              | 377              | 381              |

***Table 3. Associations of tumour pathological and molecular features with overall survival***

| Clinical variable | Tumour site | Deaths | HR (adjusted for age and gender) | 95% confidence interval of \( HR \) | \( P \)-value |
|-------------------|-------------|--------|----------------------------------|-----------------------------------|-------------|
| High HEY1         | All         | 208    | 1.29                             | 1.0–1.6                           | 0.023       |
| Colon             | 146         | 1.31   | 1.0–1.6                           | 0.037                             |
| Rectum            | 62          | 1.07   | 0.8–1.4                           | 0.664                             |
| High HES1         | All         | 206    | 1.05                             | 0.9–1.3                           | 0.596       |
| Colon             | 145         | 1.07   | 0.9–1.2                           | 0.743                             |
| Rectum            | 61          | 1.00   | 0.7–1.3                           | 0.986                             |
| High SOX9         | All         | 208    | 1.14                             | 0.9–1.4                           | 0.231       |
| Colon             | 146         | 1.17   | 0.9–1.4                           | 0.859                             |
| Rectum            | 62          | 0.97   | 0.7–1.3                           | 0.859                             |
| AJCC stage III    | All         | 541    | 1.88                             | 1.6–2.2                           | <0.001      |
| Colon             | 363         | 2.07   | 1.7–2.5                           | <0.001                            |
| Rectum            | 164         | 1.97   | 1.4–2.7                           | <0.001                            |
| High \( \beta \)-catenin | All         | 528    | 0.78                             | 0.6–1.0                           | 0.015       |
| Colon             | 356         | 0.74   | 0.6–0.9                           | 0.013                             |
| Rectum            | 157         | 0.94   | 0.7–1.4                           | 0.732                             |
| High MSI          | All         | 509    | 0.74                             | 0.5–1.0                           | 0.053       |
| Colon             | 346         | 0.70   | 0.5–1.0                           | 0.036                             |
| Rectum            | 153         | 0.93   | 0.3–4.8                           | 0.916                             |

Abbreviations: AJCC = American Joint Committee on Cancer; LN = lymph node; MSI = microsatellite instability. Protein data used to determine Spearman’s \( r \) correlation coefficients were collected at the time of colorectal cancer diagnosis. 

\*Correlation is significant at the <0.05 level (two-tailed).

\*Correlation is significant at the <0.05 level (two-tailed).

LN Metastasis is based on the ratio of positive to negative lymph nodes examined.
immunoreactivities of HEY1, HES1 and SOX9 (Figure 3). In chemotherapy-treated patients, there was an additive increase in the risk of death for each NTF highly expressed in tumours (HR = 2.09, P = 0.01). Untreated patients showed a nonsignificant trend towards reduced risk of death with overexpression of NTFs (HR = 0.74, P = 0.19).

DISCUSSION

This is the first clinical study to compare the interrelationships of HEY1, HES1 and SOX9 in CRC. We identified significant coexpression of NTFs in CRC patients. Potential causalities for this coexpression of NTFs include their mutual transcriptional activation through Notch signalling, particularly by RBP-Jk, JAG1 and NOTCH1-4, as well as their collective activation by inflammatory IL-1, TNFα and Ikκ2 signalling (Maier and Gessler, 2000; Kunnimalaiyaan et al, 2005; Staal and Langerak, 2008; Maniati et al, 2011; Wang et al, 2013). Furthermore, SOX9 is a key mediator of HES1 transcription, and retinoic acid signalling mutually promotes SOX9 and HES1 transcription (Müller et al, 2010). Hence, their clinical coexpression is consistent with our molecular understanding of NTF signalling. All three NTFs were upregulated between 3–7-fold in CRC compared with that in colonic epithelium. The upregulation of HES1 and SOX9 in tumours has been observed previously whereas HEY1 has not been studied (Reedijk et al, 2008; Matheu et al, 2012).

Tumour SOX9 and the combination of HEY1, HES1 and SOX9 protein overexpression were predictive of poorer response to chemotherapy in stage III CRC patients. Previous studies of these NTFs have never explored chemoresponse, and there exist no reports of proteins providing predictive data of this potency for adjuvant chemotherapy in CRC (Clark-Langone et al, 2010; Wang et al, 2010; Jin et al, 2011; Mann et al, 2012; Matheu et al, 2012). At the time of this study, adjuvant chemotherapy was given for stage III but not stage II disease, allowing the reduced effectiveness of chemotherapy observed with NTF overexpression to exert selective survival pressure. More recently, 5-FU and oxaliplatin combinations have become the standard of care in CRC stage III, as well as a proportion of stage II and IV patients, making the prediction of chemotherapy effectiveness by NTFs increasingly relevant to contemporary patients (DeVita et al, 2008). Furthermore, the expression of NTFs may be capable of predicting the survival of patients with other cancers that are treated with 5-FU, and hence could be studied in lung, breast, liver, stomach, oesophageal and head and neck cancer (Shirasaka and Taguchi, 2006). Tumour NTF expression also has the potential to guide the use of Notch inhibitory therapy, alone or in combination with chemotherapy to

**Table 4. Associations of HEY1, HES1 and SOX9 expression with overall survival, stratified by AJCC stage**

| Dependent variable | AJCC Stage | Deaths | HR | 95% confidence interval of HR | P-value |
|--------------------|------------|--------|----|-------------------------------|---------|
| Tumour HEY1        | II         | 115    | 1.05 | 0.8–1.4                       | 0.728   |
|                    | III        | 91     | 1.34 | 1.1–1.7                       | 0.007   |
| Tumour HES1        | II         | 106    | 0.98 | 0.8–1.2                       | 0.816   |
|                    | III        | 89     | 1.23 | 1.0–1.5                       | 0.037   |
| Tumour SOX9        | II         | 107    | 1.02 | 0.8–1.3                       | 0.829   |
|                    | III        | 90     | 1.29 | 1.0–1.6                       | 0.022   |

Abbreviations: 5-FU = 5-fluorouracil chemotherapy; AJCC = American Joint Committee on Cancer; HR = hazard ratio. Cox regression survival analyses were age and sex adjusted.

**Figure 2. Survival of chemotherapy-treated and -untreated CRC patients by HEY1, HES1 and SOX9 expression.** Survival curves for AJCC stage III CRC patients that were chemotherapy-treated based on tumour expression levels of HEY1 (A), HES1 (B) or SOX9 (C), or untreated based on tumour expression levels of HEY1 (D), HES1 (F) or SOX9 (E), grouped as high (≥1.5) or low (<1.5) by immunoreactivity. AJCC = American Joint Committee on Cancer; HR = hazard ratio.

www.bjcancer.com | DOI:10.1038/bjc.2013.431

1027
increase their benefit to patients. Hence, assuming confirmation in a larger study, the uniqueness of this data for the prognostication of adjuvant chemotherapy could see its rapid integration into current pathological practice.

Notch signalling has been implicated in chemoresistance at the preclinical level (Schreck et al., 2010; Miyamoto and Rosenberg, 2011; Izrailit and Reedijk, 2012). In CRC cells, 5-FU-, oxaliplatin- and irinotecan-induced chemoresistance was promoted by NTFs, such as HES1, and abrogated using Notch inhibitory therapy (Meng et al., 2009). Coupled with the functional roles of NTFs in apoptotic resistance and EMT (Bastide et al., 2007; Hughes, 2009; Meng et al., 2009; Kannan et al., 2011; Xie et al., 2011; Zage et al., 2011; Zhang et al., 2011; Garcia and Kandel, 2012; Matheu et al., 2012; Ueo et al., 2012), and the associations of NTF overexpression in this study with markedly poorer survival in chemotherapy-treated patients, suggests further studies that explore the clinical benefit of using combinations of Notch inhibitors and chemotherapy may lead to a reduced incidence of chemoresistance and increased survival times (Purov et al., 2005; Fan et al., 2010). In addition, the clinical limitations caused by the toxicity of existing Notch inhibitors that target upstream of the Notch signalling pathway (Staal and Langerak, 2008; Strosberg et al., 2012), to affect a broad range of targets, may be mitigated by the more targeted therapeutic inhibition of downstream NTFs.

HEY1 overexpression in tumours correlated with greater incidence of LN metastasis, perineural and vascular invasion, and poorer survival accentuated in patients with AJCC stage III CRC or colon cancer. These findings are consistent with previous studies of HEY1 in glioblastoma and pancreatic cancer (Hulleman et al., 2009; Mann et al., 2012). There may be a causal element in overexpressed HEY1’s clinical associations with increased risk of LN metastasis and mortality, because HEY1 promotes proliferation and EMT in tumours (Yang and Weinberg, 2008; Hulleman et al., 2009; Villaronga et al., 2009). HEY1 expression was associated with numerous proteins important in CRC. Firstly, overexpression of the Notch signalling target COX2 strongly correlated with HEY1, suggesting this NTF may be involved in COX2’s activation, which drives angiogenesis, inflammation and associates with reduced survival (Tseng et al., 2012; Zhou et al., 2012). HEY1 expression also correlated with Wnt pathway members β-catenin and SFRP4, an association attributable to Notch signalling’s activation of the Wnt signalling pathway (Zhang et al., 2011). Finally, HEY1 overexpression was associated with high levels of DNA-repair protein MLH1 that reduces the incidence of MSI tumours. HEY1 inversely correlated with tumours that have MSI, which has never been reported previously. This may be due to Notch signalling being more active in tumours with reduced MSI that show high Wnt signalling activity (Morikawa et al., 2011; Zhang et al., 2011). This would also explain in part the association of HEY1 with poorer survival in colon, but not rectal cancer, because MSI, a good prognostic factor, rarely occurs in rectal tumours (Minoo et al., 2010). Taken together, these data suggest HEY1 could be a therapeutic target, and its tumour expression may enhance current CRC prognostication.

HES1 overexpression in tumours correlated significantly with inferior survival in stage III CRC, consistent with trends seen in smaller CRC cohorts (Reedijk et al., 2008; Mazur et al., 2012). This association was confined to 5-FU-treated patients. HES1 overexpression correlated strongly with β-catenin’s, a Notch signalling target (Zhang et al., 2011). The association between tumour overexpressions of HES1 and master tumour suppressor P53 may be attributed to the transcriptional activation of P53 by HES1 (Huang et al., 2004). This study indicates that further research into the utility of HES1 tumour expression for the prediction of chemoresponse in CRC is warranted.

SOX9 overexpression in tumours was the strongest predictor of reduced survival in 5-FU-treated patients (HR = 8.72, P = 0.034) and correlated with vascular invasion. Previously, increased mortality associated with elevated SOX9 has been observed in numerous cancers, including separate cohorts of colon and rectal cancer (Li et al., 2008; Zhou et al., 2011; Matheu et al., 2012; Mazur et al., 2012). Many studies report mechanistic roles for SOX9 in cancer promotion, specifically in apoptotic resistance, neoplastic transformation and proliferation in glioma, colorectal and prostate cancer.
cancer (Bastide et al, 2007; Matheu et al, 2012). However, this is the first study to demonstrate value for SOX9 as a biomarker in the prediction of response to 5-FU treatment.

The most important limitation of this study is the lack of randomised chemotherapy, making any comparison between treated and untreated patients confounded by selection bias. In general, patients not receiving treatment were perceived as either at lower risk of relapse or were considered unfit for therapy. Performance status data that might have allowed a degree of compensation for the latter factor was not available. Hence, our analyses of chemoresistance have both been performed within and not between cohorts defined by receipt of therapy.

In conclusion, the present study is the first to discover that tumour HEY1 overexpression correlates with poorer CRC outcome, and NTIs may have value as biomarkers for the prediction of survival with 5-FU chemotherapy treatment. If confirmed, these NTIs, in particular SOX9, have a very real potential to enter routine clinical practice in defining the utility of adjuvant chemotherapy for individual patients. Furthermore, as there are mechanistic roles for these NTIs in CRC chemoresistance and metastasis, studies should be pursued to explore whether patients overexpressing these NTIs may benefit preferentially from Notch-targeted therapies in conjunction with chemotherapy.

ACKNOWLEDGEMENTS

We thank Barry Iacopetta and Lisa Spalding for their assistance with the tissue microarrays. This work was in part funded by the National Health and Medical Research Council (NHMRC). Patrick Candy was the recipient of a Richard Walker Gibbon Medical Research Scholarship from the University of Western Australia.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Bastide P, Darido C, Pannequin J, Kist R, Robine S, Marty-Double C, Bastide P, Darido C, Pannequin J, Kist R, Robine S, Marty-Double C, The authors declare no conflict of interest.

with the tissue microarrays. This work was in part funded by the National Health and Medical Research Council (NHMRC). Patrick Candy was the recipient of a Richard Walker Gibbon Medical Research Scholarship from the University of Western Australia.

heart defects because of impaired epithelial to mesenchymal transition. Circulation Res 100(6): 856–863.

Garcia A, Kandel J (2012) Notch: A key regulator of tumor angiogenesis and metastasis. Histol Histopathol 27(2): 151–156.

Gumbsch PM, Therneau TM (1994) Proportional hazards tests and diagnostics based on weighted residuals. Biometrika 81(3): 515–526.

Gunther K, Brablett T, Kraus C, Dworak O, Reymond M, Jung A, Hohenberger W, Kirchner T, Kockerling F, Ballhausen W (1998) Predictive value of nuclear beta-catenin expression for the occurrence of distant metastases in rectal cancer. Dis Colon Rectum 41(10): 1256–1261.

Harrell FE (2011) rms: regression modeling strategies. R Package Version 4, 3–3.

Harrell FE, Califf RM, Pryor DB, Lee KL, Rosati RA (1982) Evaluating the yield of medical tests. J Am Med Assoc 247: 2543–2546.

Huang Q, Raya A, Delesus P, Chao S-H, Quon KC, Caldwell JS, Chandra SK, Izsipusa-Belmonte JC, Schultz PG (2004) Identification of p53 regulators by genome-wide functional analysis. Proc Natl Acad Sci USA 101(10): 3456–3461.

Hughes DP (2009) How the NOTCH pathway contributes to the ability of osteosarcoma cells to metastasize. Cancer Treat Res 152: 479–496.

Hullerman EM, Quarto M, Vernell R, Masserotti G, Colli E, Kros JM, Levi D, Gaetani P, Tumini P, Finocchiaro G, Rby Buena, Capra M, Helin K (2009) A role for the transcription factor HEY1 in glioblastoma. J Cell Mol Med 13(1): 136–146.

Izrailih R, Reedijk M (2012) Developmental pathways in breast cancer and breast-tumor-initiating cells: Therapeutic implications. Cancer Letts 317(2): 115–126.

Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D (2011) Global cancer statistics. CA Cancer J Clin 61(2): 69–90.

Jin HY, Xu JH, Wang XF, Ding YJ (2011) Expression pattern and clinical significance of hairy enhancer of split 1 in colorectal cancer. Zhonghua Yi Xue Za Zhi 91(11): 2891–2894.

Kannan S, Fang W, Song G, Mullighan CG, Hammitt R, McMurray J, Zweidler-McKay PA (2011) Notch/HES1-mediated PARP1 activation: a cell type-specific mechanism for tumor suppression. Blood 117(10): 2891–2900.

Kunnimalaiyan M, Traeger K, Chen H (2005) Conservation of the Notch1 signaling pathway in gastrointestinal carcinoid cells. Am J Gastrointestinal Liver Physiol 289(4): G636–G642.

Li FY, Lai MD (2009) Colorectal cancer, one entity or three. J Zhejiang Univ Sci B 10(3): 219–229.

Li B, Fang Y, Xu J, Wang L, Xu F, Xu E, Huang Q, Lai M (2008) Analysis of SOX9 expression in colorectal cancer. Am J Clin Pathol 130(6): 897–904.

Maier MM, Gessler M (2000) Comparative analysis of the human and mouse Notch-1 signaling pathway in stage II and III colorectal cancer patients. Clin Gastroenterol Hepatol 1(1): 1017–1025.

Clark-Langone KM, Sangi C, Krishnakumar J, Watson D (2010) Translating tumor biology into personalized treatment planning: analytical performance characteristics of the Oncotype DX Colon Cancer Assay. BMC Cancer 10(691): 691.

DeVita V, Lawrence T, Rosenberg S, DePinho R, Weinberg R (2008) DeVita, Lawrence, and Rosenberg’s Cancer: Principles & Practice of Oncology. Lippincott Williams & Wilkins.

Fan X, Khaki L, Zhu TS, Soules ME, Tsalma CE, Gul N, Koh C, Zhang J, Li YM, Macizcayk J, Nikkhah G, DiMecco F, Piccirillo S, Vescovi AL, Eberhart CG (2010) NOTCH pathway blockade depletes CD133-positive glioblastoma cells and inhibits growth of tumor neurospheres and xenografts. Stem Cells 28(1): 5–16.

Feng Han Q, Zhao W, Bentel J, Shearwood A-M, Zeps N, Joseph D, Iacopetta B, Dharmarajan A (2006) Expression of SFRP-4 and [beta]-catenin in human colorectal carcinoma. Cancer Letts 231(1): 129–137.

Fischer A, Steidl C, Wagner TU, Lang E, Jakob PM, Friedl P, Knobeloch K-P, Gessler M (2007) Combined loss of Hey1 and Hey2 causes congenital
Notch targets predict colorectal cancer cheomoresponse

Morikawa T, Kuchiba A, Yamauchi M, Meyerhardt JA, Shima K, Nosho K, Chan AT, Giovannucci E, Fuchs CS, Ogino S (2011) Association of CTNNB1 (beta-catenin) alterations, body mass index, and physical activity with survival in patients with colorectal cancer. JAMA 305(16): 1685–1694.

Muto A, Iida A, Satoh S, Watanabe S (2009) The group E Sox genes Sox8 and Sox9 are regulated by Notch signaling and are required for Muller glial cell development in mouse retina. Exp Eye Res 89(4): 549–558.

Müller P, Crofts J, Newman B, Bridgewater L, Lin C-Y, Gustafsson J-Å, Muto A, Iida A, Satoh S, Watanabe S (2009) The group E Sox genes Sox8 and Sox9 mediate the retinoic acid-induced HES-1 gene expression in human breast cancer cells. Breast Cancer Res Treat 120(2): 317–326.

Purow BW, Haque RM, Noel MW, Su Q, Burdick MJ, Lee J, Sundaresan T, Pastorino S, Park JK, Mikolaenko I, Maric D, Eberhart CG, Fine HA (2005) Expression of Notch-1 and its ligands, Delta-like-1 and Jagged-1, is critical for glioma cell survival and proliferation. Cancer Res 65(6): 2353–2363.

Reedijk MOS, Zhang H, Chetty R, Tennert C, Dickson BC, Lockwood G, Gallinger SES (2008) Activation of Notch signaling in human colon adenocarcinoma. Int J Oncol 33(6): 1223–1229.

Sakamoto M, Hirata H, Ohtsuka T, Bessho Y, Kageyama R (2003) The basic helix-loop-helix genes Hes1/Hey1 and Hes2/Hey2 regulate maintenance of neural precursor cells in the brain. J Biol Chem 278(45): 44808–44815.

Salama P, Phillips M, Grieu F, Morris M, Zeps N, Joseph D, Platell C, Pastorino S, Park JK, Mikolaenko I, Maric D, Eberhart CG, Fine HA (2005) Expression of Notch-1 and its ligands, Delta-like-1 and Jagged-1, is critical for glioma cell survival and proliferation. Cancer Res 65(6): 2353–2363.

Shirasaka T, Taguchi T (2010) SOX9 mediates the retinoic acid-induced HES-1 gene expression in human breast cancer cells. Breast Cancer Res Treat 120(2): 317–326.

Stro¨m A (2010) SOX9 mediates the retinoic acid-induced HES-1 gene expression in human breast cancer cells. Breast Cancer Res Treat 120(2): 317–326.

Tseng YC, Tsai YH, Tseng MJ, Hsu KW, Yang MC, Huang KH, Li AF, Chi CW, Hsieh RH, Ku HH, Yeh YS (2012) Notch2-induced COX-2 expression enhancing gastric cancer progression. Mol Carcinog 51(12): 939–951.