MicroRNA-204 increases sensitivity of neuroblastoma cells to cisplatin and is associated with a favourable clinical outcome

J Ryan¹,2, A Tiwana¹,2, J Fay¹,2, K Bryan¹,2, M Meehan¹,2, L Creevey¹,2, J Lynch¹,2, IM Bray¹,2, A O’Meara², AM Davidoff³ and RL Stallings*¹,2

¹Department of Molecular and Cellular Therapeutics, Cancer Genetics Research Group, Royal College of Surgeons in Ireland (RCSI), York House, York Street, Dublin 2, Ireland; ²National Children’s Research Centre (NCRC), Our Lady’s Children’s Hospital, Crumlin, Dublin 12, Ireland; ³Department of Surgery, St. Jude Children’s Research Hospital, 262 Danny Thomas Place, Memphis, TN 38105-3678, USA

BACKGROUND: Neuroblastoma remains a major cause of cancer-linked mortality in children. miR-204 has been used in microRNA expression signatures predictive of neuroblastoma patient survival. The aim of this study was to explore the independent association of miR-204 with survival in a neuroblastoma cohort, and to investigate the phenotypic effects mediated by miR-204 expression in neuroblastoma.

METHODS: Neuroblastoma cell lines were transiently transfected with miR-204 mimics and assessed for cell viability using MTS assays. Apoptosis levels in cell lines were evaluated by FACS analysis of Annexin V-/propidium iodide-stained cells transfected with miR-204 mimics and treated with chemotherapy drug or vehicle control. Potential targets of miR-204 were validated using luciferase reporter assays.

RESULTS: miR-204 expression in primary neuroblastoma tumours was predictive of patient event-free and overall survival, independent of established known risk factors. Ectopic miR-204 expression significantly increased sensitivity to cisplatin and etoposide in vitro. miR-204 direct targeting of the 3’ UTR of BCL2 and NTRK2 (TrkB) was confirmed.

CONCLUSION: miR-204 is a novel predictor of outcome in neuroblastoma, functioning, at least in part, through increasing sensitivity to cisplatin by direct targeting and downregulation of anti-apoptotic BCL2. miR-204 also targets full-length NTRK2, a potent oncogene involved with chemotherapy drug resistance in neuroblastoma.

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Neuroblastoma, a paediatric cancer of the sympathetic nervous system, is responsible for 15% of all childhood cancer-associated mortalities (Brodeur, 2003). A significant hallmark of the disease is the high level of heterogeneity with clinical outcome ranging from spontaneous remission to rapid progression and death. Currently, patients are identified as high-risk according to anatomical presence of the INSS, age, histology, and the presence of genetic features, including upregulation of the MYCN oncogene (also known as TrkB). Reduced sensitivity to various chemotherapeutic drugs is mediated by high levels of the anti-apoptotic protein BCL2 (Dole et al, 1994). NTRK2, a tyrosine kinase receptor for neurotrophins such as brain-derived neurotrophic factor (BDNF), also facilitates resistance to several chemotherapeutic agents through activation of the phosphatidylinositol 3’-kinase (PI3K)/Akt pathway (Ho et al, 2002).

The identification of additional independent biomarkers associated with survival, and elucidation of their function, may offer a greater insight into the underlying biology of disease, and the mechanisms of response to treatment and relapse. MicroRNAs (miRNAs), non-coding RNA molecules that regulate gene expression at the post-transcriptional level through sequence-specific base pairing with 3’ UTRs of target mRNA, have been linked to the development of drug resistance in several cancers (Giovannetti et al, 2012). Although a role for miRNAs in neuroblastoma cell resistance to chemotherapeutic drugs has not been reported,
a number of studies have now identified miRNAs that are associated with poor clinical outcome in neuroblastoma (Chen and Stallings, 2007; Bray et al; 2009; Buckley et al, 2010; Mestdagh et al, 2010; Schulte et al, 2010), along with miRNAs that regulate a variety of processes, such as cell differentiation, apoptosis, proliferation, and invasiveness (Welch et al, 2007; Fontana et al, 2008; Foley et al, 2010, 2011; Bray et al, 2011; Lynch et al, 2012).

miR-204 has been used in miRNA expression signatures predictive of neuroblastoma patient survival (Bray et al; 2009; Schulte et al, 2010; De Preter et al, 2011). However, the independent significance of miR-204 in the outcome of neuroblastoma is unknown. Here, we report that miR-204 is a predictive determinant of outcome in neuroblastoma, with lower than median expression levels in tumours at diagnosis significantly associated with poor EFS and overall survival (OS). We demonstrate that while miR-204 has no effect in vivo or in vitro on neuroblastoma cell growth in the absence of any chemotherapeutic challenge, miR-204 increases sensitivity of neuroblastoma cell lines to cisplatin and etoposide. We demonstrate that this difference in sensitivity is due to increased levels of detectable apoptosis in miR-204-expressing cells following chemotherapy drug treatment. Finally, we determined that miR-204 directly targets the 3' UTR of both the anti-apoptotic gene BCL2 and the oncogene NTRK2 (TrkB), both of which are significantly associated with poorer patient survival in neuroblastoma through increasing resistance to treatment (Dole et al, 1994; Ho et al, 2002), thus representing at least two distinct mechanisms by which miR-204 expression is associated with increased sensitivity of neuroblastoma cells to chemotherapy and improved patient survival.

MATERIALS AND METHODS
Primary neuroblastoma tumours
In total, 143 diagnostic primary neuroblastoma tumour samples were obtained from the Children’s Oncology Group (COG), Philadelphia, PA, USA (n = 112) or from Our Lady’s Children's Hospital, Dublin, Ireland (n = 31) (Supplementary Table 1). Research was approved by the Research Ethics Committees of the Royal College of Surgeons and Our Lady’s Children’s Hospital. Detailed miRNA expression profiling of this cohort of patients is described previously (Bray et al, 2009).

Cell culture and transfections
Neuroblastoma cell lines including Kelly (MYCN amplified), SK-N-AS (non-MYCN amplified), and SHSY-5Y (non-MYCN amplified) were purchased from the European Collection of Animal Cells. MYCN-amplified NB1691 cells were obtained from Dr Andrew Davidoff (St. Jude Children’s Research Hospital, Memphis, TN, USA). All lines were valid by short tandem repeat sequence genotyping and for presence of previously published genomic imbalances using array comparative genomic hybridisation. Cell culture media was supplemented with 10% FBS and 1% Pen/Strep.

miR-204 mimics and scrambled control oligonucleotides (Ambion, Life Technologies, Carlsbad, CA, USA) were transiently transfected into neuroblastoma cells at a final concentration of 30 nM by reverse transfection using siPORT NeoFX (Ambion). Small interfering RNAs (siRNAs; siRNA negative control and siBCL2 final concentration 30 nM) (Qiagen, Crawley, UK), luciferase reporter plasmids, and miR mimics were transiently transfected into cells using LipoFectamine 2000 (Invitrogen, Carlsbad, CA, USA). Absorbance was measured at 490 nm using a Synergy Multi-Mode Plate Reader (Boitek, Winooski, VT, USA). Apoptosis levels were demonstrated by Annexin-V staining and propidium iodide (PI) exclusion using the FITC Annexin-V Apoptosis Detection Kit I (BD Pharmingen, San Diego, CA, USA). Cells were acquired using a BD LSR II flow cytometer (Becton Dickinson, San Jose, CA, USA) and analysed using BD FACSDiva 4.0 Software. Caspase 3 activity was evaluated using the Caspase-Glo 3/7 Assay (Promega) and luminescence recorded using a Synergy Multi-Mode Plate Reader (Boitek).

Animal models
Animal experiments were performed in 4-week-old CB-17/SCID mice (Charles River Laboratories, Wilmington, MA, USA) in accordance with protocols approved by the Institutional Animal Care and Use Committee of St Jude Children’s Research Hospital, Memphis, TN, USA. The orthotopic xenograft model has been previously described (Dickson et al, 2007) (Supplementary Methods 1).

Quantitative real-time RT-PCR
Total RNA was extracted from cell lines using mirNeasy Mini Kits (Qiagen, Valencia, CA, USA). Reverse transcription was performed using total RNA with primers specific for miR-204 or U48 control and TaqMan miRNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). For gene expression analysis, reverse transcription was performed using High-Capacity reverse transcription kits (Applied Biosystems). Specific TaqMan assays (Applied Biosystems) for BCL2, NTRK2, and miR-204 were used for expression analysis on the 7900 HT Fast Realtime System (Applied Biosystems). MicroRNA and gene expression was normalised using the endogenous controls RNU48 and 18S, respectively, and relative quantities determined by the delta CT method.

Western blot analysis
Total protein was analysed by western blotting using primary antibodies anti-TrkB (G11) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-BCL2 (Cell Signaling Technology, Beverly, MA, USA), followed by anti-mouse and anti-rabbit IgG HRP-conjugated secondary antibodies (Cell Signalling Technology).

Luciferase reporter assay
Direct targeting of 3' UTRs was determined by cloning of the 3'-UTR seed region and a mutated seed region into separate psiCHECK-2 vectors (Eurofins MWG Operon, Anzingernstr, Ebersberg, Germany) for both BCL2 and NTRK2. Renilla and firefly luciferase activities were measured using the Dual-Luciferase Reporter Kit (Promega) and luminescence recorded on a Synergy Multi-Mode Plate Reader (Boitek).

Statistical analysis
All statistical analyses were performed using GraphPad prism 5 software (GraphPad Software, San Diego, CA, USA) or MedCalc Version 12.2.1.0 (MedCalc Software, Mariakerke, Belgium). A P-value of < 0.05 was regarded as statistically significant.

RESULTS
miR-204 expression is predictive of patient event-free and overall survival, independent of known risk factors
Analysis of miR-204 expression levels in 143 primary diagnostic neuroblastoma samples (Supplementary Table 1) revealed significantly
miR-204 expression is significantly associated with neuroblastoma disease subtypes and patient survival. (A) Box and whiskers plots represent the expression of miR-204 in a cohort of 143 primary neuroblastoma tumours. Samples are grouped according to known risk factors and miR-204 expression were analysed using two-sided Mann–Whitney U tests. Dark shading indicates known higher-risk factor. (B) Expression of miR-204 in higher-risk INSS stages 3 and 4 vs lower-risk stages 1, 2, 4S, and in high-risk INSS stage 4 vs stage 1, 2, 3, 4S tumours. Samples are further grouped according to the presence of MYCNA and/or 11q LOH (MYCNA/11q-) and non-MYCNA and 11q LOH (designated as ‘other’). (C, D) Kaplan–Meier plots for EFS and OS in 143 neuroblastoma patients, with miR-204 expression > or < median. P-values were obtained using log-rank test. (E) Kaplan–Meier plots for EFS in high-risk INSS stage 4 disease (n = 72) with miR-204 expression > or < median. (F) Kaplan–Meier plots for EFS in lower-risk INSS stage 1, 2, 3, 4S disease (n = 71) with miR-204 expression > or < median. Abbreviation: HR = hazard ratio.

In univariate survival analysis, miR-204 was significantly associated (log-rank \( P < 0.0001 \)) with both EFS and OS, with > median expression of miR-204 indicative of improved survival (EFS HR 4.9, 5-year EFS 34.5% vs 80.3%; OS HR 5.6, 5-year OS 41.5% vs 81.6%) (Figure 1C and D). Multivariate Cox regression analysis of time to EFS excluded the variables of patient age, MYCN status, and 11q status in a stepwise prediction model of lower expression of miR-204 in patients with known higher-risk prognostic factors including MYCN amplification, 11q deletion, and INSS stage 3 or 4 disease (Figure 1A). In samples stratified according to INSS stage, the expression of miR-204 remained significantly lower in stage 3 and/or stage 4 tumours irrespective of MYCN or 11q status, consistent with an independent predictive power (Figure 1B).
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Table I Univariate (Kaplan–Meier) and multivariate (Cox proportional hazard regression) analysis of event-free survival in 143 neuroblastoma patients

| Variable | n  | HR  | 95% CI | EFS P | Entered | HR  | 95% CI | EFS P | Stepwise | HR  | 95% CI | EFS P |
|----------|----|-----|--------|-------|---------|-----|--------|-------|----------|-----|--------|-------|
| INSS     |    |     |        |       |         |     |        |       |          |     |        |       |
| Stage 4  | 72 | 6.59| 3.3–12.9| <0.0001 |         | 3.10| 1.3–7.3| 0.0095 |          | 5.25| 2.4–11.4| <0.0001 |
| Stage 1, 2, 3, 45 | 71 | 1 |       |       |         |     |        |       |          |     |        |       |
| Stage 3, 4 | 104 | 22.5 | 11.7–43.1| <0.0001 |         | 6.49| 0.8–52.6| 0.0812 |          |     |        |       |
| Stage 1, 2, 45 | 39 | 1 |       |       |         |     |        |       |          |     |        |       |
| Age      |    |     |        |       |         |     |        |       |          |     |        |       |
| ≥ 1 year | 123 | 2.42| 1.1–5.4| 0.0331 |         | 2.69| 0.6–11.7| 0.1815 |          |     |        |       |
| < 1 year | 20  | 1 |       |       |         |     |        |       |          |     |        |       |
| MYCN     |    |     |        |       |         |     |        |       |          |     |        |       |
| Amplified| 33 | 3.7 | 1.7–8.1| 0.0011 |         | 1.80| 0.9–3.8| 0.1187 |          |     |        |       |
| Non amplified | 110 |    |       |       |         |     |        |       |          |     |        |       |
| 11q Deletion | 50 | 2.25| 1.3–4.4| 0.0154 |         | 1.28| 0.6–2.7| 0.0132 |          |     |        |       |
| No deletion | 93 |    |       |       |         |     |        |       |          |     |        |       |
| MiR-204  |    |     |        |       |         |     |        |       |          |     |        |       |
| Low expression | 72 | 4.89| 2.5–9.3| <0.0001 |         | 2.61| 1.1–5.8| 0.0206 |          |     | 3.13 | 1.69–7.9| 0.0011 |
| High expression | 71 |    |       |       |         |     |        |       |          |     |        |       |

Abbreviations: EFS = event-free survival; HR = hazard ratio; INSS = international neuroblastoma staging system; 95% CI = 95% confidence interval. *Kaplan–Meier P = values for EFS = log-rank. ‡Variables entered in Cox proportional hazard model: miR-204 expression (low expression < median, high expression > median), INSS (stage 3 and 4, stages 1, 2, 45 and stage 4, stages 1, 2, 3, 45), age (≥ 1 year, < 1 year), MYCN amplified (yes, no) and chromosome 11q deleted (yes, no). ‡The variables age, MYCN status, 11q status and INSS stage 3, 4 vs stage 1, 2, 45 were found not to significantly contribute to the prediction of EFS time and were excluded in the stepwise Cox multivariate model.

Ectopic miR-204 expression increases sensitivity of neuroblastoma cells to cisplatin in vitro

Given the significant association of low miR-204 expression with poor patient survival, we investigated the effects on viability of miR-204 ectopic expression in neuroblastoma cells by transiently transfecting mature miR-204 mimics into Kelly, NB1691, SK-N-AS, and SHSY-5Y cell lines. Although miR-204 expression was significantly upregulated following transfection in all cell lines (Supplementary Figure 1), we observed no discernable effect on cell viability, as determined by MTS assays, relative to negative controls for both cell lines (mean differences in cell viability; Kelly 8.94 ± 2.9% P = 0.001, NB1691 8.2 ± 2.6% P = 0.001) (Figure 2A and B). These observations were confirmed in further independent experiments using 5 µg ml⁻¹ CDDP treatment (mean differences in cell viability; Kelly 8.94 ± 2.9% P = 0.001, NB1691 8.2 ± 2.6% P = 0.001) (Figure 2C and D).

Through in silico analysis (using the algorithms PicTar, TargetScan, and mirRanda), we examined all computationally predicted target genes of miR-204. There were 107 targets common to all three algorithms (Supplementary Figure 3 and Supplementary Table 2). BCL2 was a target of interest owing to its known role in increased resistance to CDDP-triggered apoptosis in neuroblastoma. To test the hypothesis that miR-204 targeting of BCL2 may account for the observed increase in sensitivity to CDDP, we performed siRNA-mediated inhibition of BCL2 in parallel with miR-204 overexpression. BCL2 was significantly reduced at mRNA and protein levels following siBCL2 transfection (Supplementary Figure 4). In the absence of CDDP treatment, BCL2 knockdown resulted in significantly decreased survival of Kelly cells, but not NB1691 cells (Figure 2C and D). However, treatment of both cell lines with 5 µg ml⁻¹ CDDP, following siRNA knockdown of BCL2, resulted in significantly decreased cell viability relative to negative controls for both cell lines (mean differences; Kelly 14 ± 2.0% P = 0.0001; NB1691 14.6 ± 2.9% P = 0.0001), similar to the response seen in cells overexpressing miR-204 (Figure 2C and D).

To confirm that siRNA-mediated inhibition of BCL2 enhanced CDDP-mediated apoptosis, and to investigate if decreased viability owing to overexpression of miR-204 was due to a similar increase in apoptotic levels, caspase 3/7 and Annexin V/Pi assays were performed. Kelly cells transfected with either miR-204 mimics or siBCL2 displayed significantly increased caspase 3/7 activation within 72 h post transfection and following treatment with 5 µg ml⁻¹ CDDP (Figure 3A), consistent with results from MTS viability experiments. Apoptosis levels determined by Annexin V/Pi staining and FACs analysis confirmed that the observed increases in
Ectopic expression of miR-204 in neuroblastoma cell lines increases sensitivity to cisplatin (CDDP) in vitro. (A, B) Neuroblastoma MYCN-amplified cell lines Kelly and NB1691 were transfected with miR-204 mimics or scrambled negative control (NC) oligonucleotides. At 48 h post transfection, cells were treated for 24 h with 0 to 50 μg ml⁻¹ CDDP. Media were replaced for a further 24 h, and viability determined at 96 h by MTS assays. (C, D) Kelly and NB1691 cells were transfected with an siRNA targeting BCL2 or a siRNA negative control oligonucleotide (siNEG NC), or miR-204 mimics or scrambled negative control (scrambled NC) oligonucleotides. At 48 h post transfection, cells were treated for 24 h with or without 5 μg ml⁻¹ CDDP. Media were replaced for a further 24 h, and viability determined at 96 h by MTS assays. Percentage survival was determined relative to either untreated siNEG NCs or untreated scrambled NCs. All graphed values represent (mean value ± s.e.m.) biological replicate experiments (n = 3–5) with six technical replicates, and differences in viability were analysed by two-sided Student’s t-tests.

miR-204 targets BCL2 and TrkB in neuroblastoma cell lines

Given that siRNA-mediated inhibition of BCL2, a computationally predicted target of miR-204, had a similar phenotypic effect on CDDP drug sensitivity as miR-204 overexpression, we decided to experimentally validate BCL2 as a miR-204 direct target. BCL2 has a highly conserved 7mer seed match with miR-204 in its 3’ UTR (Figure 4A). Ectopic overexpression of miR-204 mimics in Kelly cells resulted in a knockdown of BCL2 protein but not mRNA, indicating that the miRNA had a potential inhibitory effect on translation (Figure 4B).

To determine if miR-204 directly targets the 3’ UTRs of BCL2, luciferase reporter plasmids were constructed containing a 450-bp segment of the BCL2 3’ UTR with either the wild-type or a mutated miR-204 seed site (Supplementary Figure 7). Cotransfection of the reporter construct containing the wild-type-binding sequence with mature miR-204 mimics resulted in a statistically significant reduction in luciferase activity in Kelly cells (Figure 4C). This effect was abrogated by a mutated target sequence, thereby confirming that BCL2 is directly targeted by miR-204.

Another computationally predicted target of interest was NTRK2, a tyrosine kinase receptor for several neurotrophins such as BDNF, NT-3, and NT-4. The full-length NTRK2 mRNA is expressed at higher levels in unfavourable tumour subtypes (Matsumoto et al., 1995), and NTRK2 protein (with intact tyrosine kinase domain) increases resistance of neuroblastoma cells to chemotherapeutic drugs, including CDDP (Ho et al., 2002; Jaboin et al., 2002). NTRK2 has one highly conserved 7mer seed match and two poorly conserved 7mer matches with miR-204 in its 3’ UTR (Figure 4D).

Analysis of the impact of miR-204 on endogenous levels of NTRK2 mRNA and protein in neuroblastoma cells was complicated by the fact that the full-length NTRK2 is rarely detected in neuroblastoma...
cell lines, even though this variant is usually expressed at high levels in unfavourable tumour subtypes. To overcome this, we stimulated the cells for 48 h with 5 μM all-trans retinoic acid (ATRA) to induce the expression of full-length NTRK2 (Middlemas et al., 1999) following transfection of SHSY-5Y cells with miR-204 mimics. Ectopic expression of miR-204 resulted in significant knockdown of NTRK2 at both mRNA and protein levels following expressional induction by ATRA (Figure 4E).

Luciferase reporter plasmids containing a 499-bp segment of the NTRK2 3' UTR with either the wild-type or a mutated 8mer seed site for miR-204 were then used to determine if this miRNA directly targets NTRK2 (Supplementary Figure 7). Cotransfection of the reporter construct containing the wild-type-binding sequence with mature miR-204 mimics resulted in a statistically significant reduction in luciferase activity in Kelly cells (Figure 4F). This effect was abrogated by a mutated target sequence, thereby confirming that NTRK2 is directly targeted by miR-204.

Owing to the lack of full-length NTRK2 expression in our cell lines, we were aware that our observed effects of miR-204 on

Figure 3  miR-204 increases apoptotic levels of neuroblastoma cells following treatment with CDDP. Kelly cells were transfected with siRNA targeting BCL2 or siRNA negative control oligonucleotide (siNEG NC), or miR-204 mimics or scrambled negative control (scrambled NC) oligonucleotides. At 48 h post transfection, cells were treated for 24 h with or without 5 μg ml⁻¹ CDDP. Graphed data are mean values ± s.e.m. of at least three biological replicate experiments. P-values were determined using two-sided Student’s t-tests. (A) Caspase 3/7 activation was measured at 72 h post transfection (n = 3, with five technical replicates). (B) Mean percentage of annexin V-positive Kelly cells from at least three independent experiments at 72 h post transfection with siBCL2/siNEG NC, or miR-204 mimic/scrambled NC and with/without 5 μg ml⁻¹ CDDP treatment. (C) Representative scatter plots of PI vs Annexin V-FITC staining in one independent experiment with Kelly cells at 72 h.
BCL2 and NTRK2 are direct targets of miR-204. Kelly cells were transfected with miR-204 mimics or scrambled negative control (scrambled NC) oligonucleotides. Graphed data are mean values ± s.e.m. of at least three independent experiments. (A) Conserved 8-mer seed match with hsa-miR-204 in BCL2 3′ UTR. (B) BCL2 mRNA relative expression to controls was determined by RQ-PCR at 24, 72 and 96 h post transfection, and BCL2 protein levels determined by western blot following total protein extraction at 48 h. (C) Kelly cells were cotransfected with miR-204 mimics or scrambled NC and either wild-type (WT) or mutated (mut) BCL2 3′ UTR reporter constructs. Luciferase activity was determined 48 h post transfection. (D) Conserved 8-mer seed match with hsa-miR-204 in NTRK2 3′ UTR. (E) Following transfection, Kelly cells were treated with 5 μM ATRA for 48 h to induce full-length NTRK2 expression. NTRK2 mRNA expression relative to controls was determined by RQ-PCR at 72 and 96 h post transfection, and NTRK2 protein levels determined by western blot following total protein extraction at 72 h. (F) Kelly cells were cotransfected with miR-204 mimics or scrambled NC and either WT or mutant NTRK2 3′-UTR reporter constructs. Luciferase activity was determined 48 h post transfection.

Figure 4  BCL2 and NTRK2 are direct targets of miR-204. Kelly cells were transfected with miR-204 mimics or scrambled negative control (scrambled NC) oligonucleotides. Graphed data are mean values ± s.e.m. of at least three independent experiments. (A) Conserved 8-mer seed match with hsa-miR-204 in BCL2 3′ UTR. (B) BCL2 mRNA relative expression to controls was determined by RQ-PCR at 24, 72 and 96 h post transfection, and BCL2 protein levels determined by western blot following total protein extraction at 48 h. (C) Kelly cells were cotransfected with miR-204 mimics or scrambled NC and either wild-type (WT) or mutated (mut) BCL2 3′-UTR reporter constructs. Luciferase activity was determined 48 h post transfection. (D) Conserved 8-mer seed match with hsa-miR-204 in NTRK2 3′ UTR. (E) Following transfection, Kelly cells were treated with 5 μM ATRA for 48 h to induce full-length NTRK2 expression. NTRK2 mRNA expression relative to controls was determined by RQ-PCR at 72 and 96 h post transfection, and NTRK2 protein levels determined by western blot following total protein extraction at 72 h. (F) Kelly cells were cotransfected with miR-204 mimics or scrambled NC and either WT or mutant NTRK2 3′-UTR reporter constructs. Luciferase activity was determined 48 h post transfection.

sensitivity to CDDP in neuroblastoma cells in vitro were not contributed to NTRK2 targeting in these cell lines. However, NTRK2 is associated with poor survival in neuroblastoma by promoting cell survival and chemotherapy resistance through the PI3K/AKT pathway, which is a well-established mechanism of resistance in neuroblastoma patients. We conclude that miR-204 directly targets the 3′ UTR of both BCL2 and NTRK2, two distinct and known mechanisms of increased resistance to chemotherapy in neuroblastoma.

DISCUSSION

Resistance to conventional chemotherapy remains a significant obstacle in the successful treatment of high-risk neuroblastoma, and for the most part the mechanisms of relapse and resistance remain poorly understood. In this report, we provide the first evidence of the role of miR-204 in increasing cancer cell sensitivity to chemotherapeutic drugs, providing a biological rationale for why lower expression of this miRNA in neuroblastoma patient tumours is significantly associated with poor survival. Here, we have identified and experimentally validated two novel targets of miR-204, NTRK2 and BCL2, both encoding cellular pro-survival effectors that are involved with increased resistance to chemotherapy in neuroblastoma. While targeting of BCL2 by several different miRNAs including miR-497, miR-181b, miR-15b, and miR-16 and the role in chemoresistance has been well described in different cancers, to our knowledge miRNA targeting of full-length NTRK2 has not been previously observed (Xia and Zhang, 2008; Lam et al, 2010; Zhu et al, 2010, 2012).

The characterisation of miR-204 functions, to date, has been limited, although several mRNA targets have been identified that are important in normal cell development, including MEIS1, HOXA9, MEIS2, RUNX2, and SIRT1 (Garzon et al, 2008; Conte et al, 2010; Huang et al, 2010; Saunders et al, 2010). miR-204 has been reported to act as a tumour suppressor in a variety of cancers through different mechanisms, including suppression of tumour growth via inhibition of macroautophagy in renal clear cell carcinoma, and suppression of invasion in endometrial cancer and squamous cell carcinoma (Lee et al, 2010; Chung et al, 2012; Mikhailova et al, 2012). In the case of endometrial cancer, miR-204 was determined to regulate cell migration and invasion.
by targeting the FOXC1 gene. Similarly, this miRNA reduced migration, invasion, and the formation of metastatic tumours in models of squamous cell carcinoma but had no effect on proliferation or viability. In contrast, a recent publication by Gong et al. (2012) demonstrated that miR-204 expression in malignant peripheral nerve sheath tumour significantly reduced cellular proliferation in vitro.

Considering our observed results, a recent study of particular interest was by Li et al. (2011) who suggested that miR-204 might contribute to the regulation of cell survival under stress conditions in human trabecular meshwork cells through validated targeting of the anti-apoptotic proteins BCL2L2 and BIRC2. With regard to this role of miR-204 in regulating cell survival, they previously demonstrated that miR-204 overexpression in cholangiocarcinoma cell lines resulted in significant reduction of detectable BCL2 protein levels, although direct targeting of miR-204 with the 3′ UTR of BCL2 was not demonstrated (Chen et al., 2009).

BCL2 has a critical role in ensuring the survival of early developing cell types and is strongly expressed in the developing sympathetic nervous system (Bashamboo et al., 2006; Hoehner et al., 1998). Castle et al. (1993) determined that BCL2 protein was detectable in approximately a third of neuroblastoma tumours at diagnosis, and in >80% of tumours following treatment, and was significantly associated with poor prognosis. Furthermore, the same group determined that BCL2 association with poor survival results from increased resistance to chemotherapy-induced apoptosis in neuroblastoma previously demonstrated by targeting the anti-apoptotic proteins NTRK2 and BCL2. The investigation of additional targets of miR-204, which may further explain a tumour suppressor role in neuroblastoma, is warranted. The upregulation of miR-204 in tumours could be envisioned as a potential method for increasing sensitivity of neuroblastoma cells to chemotherapy in vivo. In this regard, we have recently demonstrated nanoparticle-mediated targeted delivery of tumour suppressive miRNA to orthotopic xenograft tumours in a murine disease model (Tivnan et al., 2012). Further studies of miR-204 as a potential prognostic marker in a much larger tumour cohort is also warranted and might eventually allow for more refined therapy stratification.

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REFERENCES

Bashamboo A, Taylor AH, Samuel K, Panthier JJ, Whetton AD, Forrester LM (2006) The survival of differentiating embryonic stem cells is dependent on the SCF-KIT pathway. J Cell Sci 119: 3039–3046

Bender A, Opel D, Naumann I, Kappler D, Friedman L, von Schweinitz D, Bray I, Bryan K, Prenter S, Buckley PG, Foley NH, Murphy DM, Alcock L, Higgins DG, O’Meara A, O’Sullivan M, Stallings RL (2009) Widespread demethylation of miR-204 expression in neuroblastoma. British Journal of Cancer (2012)

Brodeur GM (2003) Neuroblastoma: biological insights into a clinical enigma. Nat Rev Cancer 3: 203–216

Buckley PG, Alcock L, Bryan K, Bray I, Schulte JH, Schramm A, Eggert A, Mestdagh P, De Preter K, Vandesompele J, Speleman F, Stallings RL (2010) Chromosomal and microRNA expression patterns reveal biologically distinct subgroups of 11q- neuroblastoma. Clin Cancer Res 16: 2971–2978

Castle VP, Heidelberger KP, Bromberg J, Ou X, Dole M, Nunez G (1993) Expression of the apoptosis-suppressing protein bcl-2, in neuroblastoma is associated with unfavorable histology and N-myc amplification. Am J Pathol 143: 1543–1550

Charlet J, Schneekenburger M, Brown KW, Diederich M (2012) DNA demethylation increases sensitivity of neuroblastoma cells to chemotherapeutic agents. Biochem Pharmacol 83: 858–865

Chen L, Yan HY, Yang W, Hu L, Yu LX, Liu Q, Li L, Huang DD, Ding J, Shen F, Zhou WP, Wu MC, Wang HY (2009) The role of microRNA: MicroRNA-204 increases sensitivity of neuroblastoma cells J Ryan et al. PLoS One 4: e7850

Bray I, Tivnan A, Bryan K, Foley NH, Watters KM, Tracey L, Davidoff AM, Stallings RL (2011) MicroRNA-542-3p as a novel tumor suppressor in neuroblastoma. Cancer Lett 309: 56–64

In conclusion, miR-204 has emerged as an independent predictor of survival in neuroblastoma, functioning, at least in part, through direct targeting and downregulation of key chemotherapy resistance proteins NTRK2 and BCL2. The investigation of additional targets of miR-204, which may further explain a tumour suppressor role in neuroblastoma, is warranted. The upregulation of miR-204 in tumours could be envisioned as a potential method for increasing sensitivity of neuroblastoma cells to chemotherapy in vivo. In this regard, we have recently demonstrated nanoparticle-mediated targeted delivery of tumour suppressive miRNA to orthotopic xenograft tumours in a murine disease model (Tivnan et al., 2012). Further studies of miR-204 as a potential prognostic marker in a much larger tumour cohort is also warranted and might eventually allow for more refined therapy stratification.

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REFERENCES

Bashamboo A, Taylor AH, Samuel K, Panthier JJ, Whetton AD, Forrester LM (2006) The survival of differentiating embryonic stem cells is dependent on the SCF-KIT pathway. J Cell Sci 119: 3039–3046

Bender A, Opel D, Naumann I, Kappler D, Friedman L, von Schweinitz D, Bray I, Bryan K, Prenter S, Buckley PG, Foley NH, Murphy DM, Alcock L, Higgins DG, O’Meara A, O’Sullivan M, Stallings RL (2009) Widespread demethylation of miR-204 expression in neuroblastoma. British Journal of Cancer (2012)

Brodeur GM (2003) Neuroblastoma: biological insights into a clinical enigma. Nat Rev Cancer 3: 203–216

Buckley PG, Alcock L, Bryan K, Bray I, Schulte JH, Schramm A, Eggert A, Mestdagh P, De Preter K, Vandesompele J, Speleman F, Stallings RL (2010) Chromosomal and microRNA expression patterns reveal biologically distinct subgroups of 11q- neuroblastoma. Clin Cancer Res 16: 2971–2978

Castle VP, Heidelberger KP, Bromberg J, Ou X, Dole M, Nunez G (1993) Expression of the apoptosis-suppressing protein bcl-2, in neuroblastoma is associated with unfavorable histology and N-myc amplification. Am J Pathol 143: 1543–1550

Charlet J, Schneekenburger M, Brown KW, Diederich M (2012) DNA demethylation increases sensitivity of neuroblastoma cells to chemotherapeutic agents. Biochem Pharmacol 83: 858–865

Chen L, Yan HY, Yang W, Hu L, Yu LX, Liu Q, Li L, Huang DD, Ding J, Shen F, Zhou WP, Wu MC, Wang HY (2009) The role of microRNA
expression pattern in human intrahepatic cholangiocarcinoma. J Hepatol 50: 358–369

Chen Y, Stallings RL (2007) Differential patterns of microRNA expression in neuroblastoma are correlated with prognosis, differentiation, and apoptosis. Cancer Res 67: 976–983

Chung TK, Lau TS, Cheung TH, Yim SF, Lo KW, Siu NS, Chan LK, Yu MY, Kwok JY, Doring LM, Ng AS, Wong RW, Wang VW, Mok SC, Smith DI, Berkowitz RS, Wong YF (2012) Dysregulation of microRNA-204 mediates migration and invasion of endometrial cancer by regulating FOXL1. Int J Cancer 130: 1036–1045

Cohn SL, Pearson AD, London MM, Zhou J, Hargrove PW, McCarrville MB, Davidoff AM (2007) In vivo bioluminescence imaging for early detection and monitoring of disease progression in a murine model of neuroblastoma. J Pediatr Surg 42: 1172–1179

Dole M, Nunez G, Merchant AK, Maybaum J, Rode CK, Bloch CA, Castle VP (1994) Bcl-2 inhibits chemotherapeutic-induced apoptosis in neuroblastoma. Cancer Res 54: 3253–3259

Foley NH, Bray IM, Tivnan A, Bryan K, Murphy DM, Buckley PG, Ryan J, Fontana L, Fiori ME, Albini S, Cifaldi L, Giovinazzi S, Forloni M, Boldrini E, Falini B, Erazenci A, Danesi R, Peters GJ (2012) Molecularly critically regulates carcinogenesis in malignant peripheral nerve sheath tumors (2012).

Falini B (2008) Distinctive microRNA signature of acute myeloid leukemia bearing cytoplasmic mutated nucleophosmin.

Fontana L, Fiori ME, Albini S, Cifaldi L, Giovinazzi S, Forloni M, Boldrini E, Falini B, Erazenci A, Danesi R, Peters GJ (2012) Molecularly critically regulates carcinogenesis in malignant peripheral nerve sheath tumors (2012).

Huang J, Zhao L, Xing L, Chen D (2010) MicroRNA-204 regulates Runx2 tissues.

Ho R, Eggert A, Lahmann C, Porro A, Haber M, Diolaiti D, Iraci N, Henderson M, Gherardi S, Valli E, Schulte JH, Verdin E (2010) miR-204 to suppress head and neck tumor metastasis. PLoS Comput Biol 6: e1000730

Lynch J, Fay J, Meehan M, Bryan K, Watters KM, Murphy DM, Stallings RL (2012) MiRNA-335 suppresses neuroblastoma cell invasiveness by direct targeting of multiple genes from the non-canonical TGF-beta signalling pathway. Carcinogenesis 33: 976–985

Manchop CF, Bray JA, Salwen HR, Madafijlo J, Cheng A, Flemming C, Marshall GM, Norris MD, Haber M, Cohn SL (2004) MYCN-mediated regulation of the MRPL promoter in human neuroblastoma. Oncogene 23: 753–762

Marras JM (2010) Recent advances in neuroblastoma. N Engl J Med 362: 2202–2211

Marras JM, Hoyart MD, Bagatell R, Cohn SL (2007) Neuroblastoma. Lancet 369: 2178–2190

Matsuomo K, Wada RK, Yamashiro JM, Kaplan DR, Thiele CJ (1995) Expression of brain-derived neurotrophic factor and p145TrkB affects survival, differentiation, and invasiveness of human neuroblastoma cell lines. Cancer Res 55: 1729–1730

Mestdagh A, Bagatell R, Cohn SL (2007) Neuroblastoma. Lancet 369: 2178–2190

Miyakawa Y, Sano H, Taniguchi T, Yamaguchi M, Nakanishi Y, Shimizu S, Nakajima K, Hasebe T, Kanzaki T, Takahashi T, Minami T, Takahashi M, Endo T, Yonezawa M, Hasegawa T, Nakanishi Y, Tanaka T, Honda H, Moriyama T, Sato S, Miyagawa Y, Nakanishi Y, Tanaka T, Honda H, Moriyama T, Sato S, Miyagawa Y, Nakanishi Y, Tanaka T, Honda H, Moriyama T, Sato S, Miyagawa Y, Nakanishi Y, Tanaka T, Honda H, Moriyama T, Sato S, Miyagawa Y, Nakanishi Y, Tanaka T, Honda H, Moriyama T, Sato S, Miyagawa Y, Nakanishi Y, Tanaka T, Honda H, Moriyama T, Sato S, Miyagawa Y, Nakanishi Y, Tanaka T, Honda H, Moriyama T, Sato S, Miyagawa Y, Nakanishi Y, Tanaka T, Honda H, Moriyama T, Sato S, Miyagawa Y, Nakanishi Y, Tanaka T, Honda H, Moriyama T, Sato S, Miyagawa Y, Nakanishi Y, Tanaka T, Honda H, Moriyama T, Sato S, Miyagawa Y, Nakanishi Y, Tanaka T, Honda H, Moriyama T, Sato S, Miyagawa Y, Nakanishi Y, Tanaka T, Honda H, Moriyama T, Sato S, Miyagawa Y, Nakanishi Y, Tanaka T, Honda H, Moriyama T, Sato S, Miyagawa Y, Nakanishi Y, Tanaka T, Honda H, Moriyama T, Sato S, Miyagawa Y, Nakanishi Y, Tanaka T, Honda H, Moriyama T, Sato S, Miyagawa Y, Nakanishi Y, Tanaka T, Honda H, Moriyama T, Sato S, Miyagawa Y, Nakanishi Y, Tanaka T, Honda H, Moriyama T, Sato S, Miyagawa Y, Nakanishi Y, Tanaka T, Honda H, Moriyama T, Sato S, Miyagawa Y, Nakanishi Y, Tanaka T, Honda H, Moriyama T, Sato S, Miyagawa Y, Nakanishi Y, Tanaka T, Honda H, Moriyama T, Sato S, Miyagawa Y, Nakanishi Y, Tanaka T, Honda H, Moriyama T, Sato S, Miyagawa Y, Nakanishi Y, Tanaka T, Honda H, Moriyama T, Sato S, Miyagawa Y, Nakanishi Y, Tanaka T, Honda H, Moriyama T, Sato S, Miyagawa Y, Nakanishi Y, Tanaka T, Honda H, Moriyama T, Sato S, Miyagawa Y, Nakanishi Y, Tanaka T, Honda H, Moriyama T, Sato S, Miyagawa Y, Nakanishi Y, Tanaka T, Honda H, Moriyama T, Sato S, Miyagawa Y, Nakanishi Y, Tanaka T, Honda H, Moriyama T, Sato S, Miyagawa Y, Nakanishi Y, Tanaka T, Honda H, Moriyama T, Sato S, Miyagawa Y, Nakanishi Y, Tanaka T, Honda H, Moriyama T, Sato S, Miyagawa Y, Nakanish
MicroRNA-204 increases sensitivity of neuroblastoma cells

Welch C, Chen Y, Stallings RL (2007) MicroRNA-34a functions as a potential tumor suppressor by inducing apoptosis in neuroblastoma cells. *Oncogene* 26: 5017–5022

Xia L, Zhang D, Du R, Pan Y, Zhao L, Sun S, Hong L, Liu J, Fan D (2008) miR-15b and miR-16 modulate multidrug resistance by targeting BCL2 in human gastric cancer cells. *Int J Cancer* 123: 372–379

Yu AL, Gilman AL, Ozkaynak MF, London WB, Kreissman SG, Chen HX, Smith M, Anderson B, Villablanca JG, Matthay KK, Shimada H, Grupp SA, Seeger R, Reynolds CP, Buxton A, Reisfeld RA, Gillies SD, Cohn SL, Maris JM, Sondel PM, Children’s Oncology Group (2010) Anti-GD2 antibody with GM-CSF, interleukin-2, and isotretinoin for neuroblastoma. *N Engl J Med* 363: 1324–1334

Zhu W, Shan X, Wang T, Shu Y, Liu P (2010) miR-181b modulates multidrug resistance by targeting BCL2 in human cancer cell lines. *Int J Cancer* 127: 2520–2529

Zhu W, Xu H, Zhu D, Zhi H, Wang T, Wang J, Jiang B, Shu Y, Liu P (2012) miR-200b/c/429 cluster modulates multidrug resistance of human cancer cell lines by targeting BCL2 and XIAP. *Cancer Chemother Pharmacol* 69: 723–731

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