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

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Translational Therapeutics

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 disease at diagnosis (international neuroblastoma staging system, INSS), age, histology, and the presence of genetic features, including amplification of the *MYCN* oncogene, chromosome 11q deletion, and diploidy (Maris *et al*, 2007; Cohn *et al*, 2009; Maris, 2010). Although novel therapeutic approaches such as anti-GD2 immunotherapy have improved patient survival rates (2-year event-free survival (EFS) of 66% reported in high-risk neuroblastoma), 5-year survival rates are currently not available and this therapy has considerable deleterious side effects (Yu *et al*, 2010).

Although less favourable tumours initially respond well to induction chemotherapy therapy, the relapse rate is high, with relapse disease usually refractory to further treatment. Initial treatment may facilitate the selection of more resistant subclones existing within the population, but the mechanisms of resistance and relapse in neuroblastoma remain for the large part poorly understood. Evidence suggests that increased drug efflux through upregulation of ATP-binding transporter genes (often under the regulation of MYCN) represents one mechanism of resistance in high-risk neuroblastoma (Manohar *et al*, 2004; Porro *et al*, 2010). Epigenetic silencing through DNA hypermethylation of tumour suppressor genes in neuroblastoma has also been demonstrated as a mechanism of increased resistance to chemotherapy drugs (Teitz *et al* 2000; Charlet *et al*, 2012). Distinct specific mechanism of resistance in neuroblastoma include enhanced expression of *BCL2* and *NTRK2* (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 neuro-trophic factor (BDNF), also facilitates resistance to several chemotherapeutic agents through activation of the phosphatidyl-inositol 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 where by 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 validated 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).

Cell viability and apoptosis assays

Viability of cells was measured by MTS-formazan reduction using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) at 24, 48, 72, and 96 h post transfection.

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 microRNA 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, Anzingerstr, 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
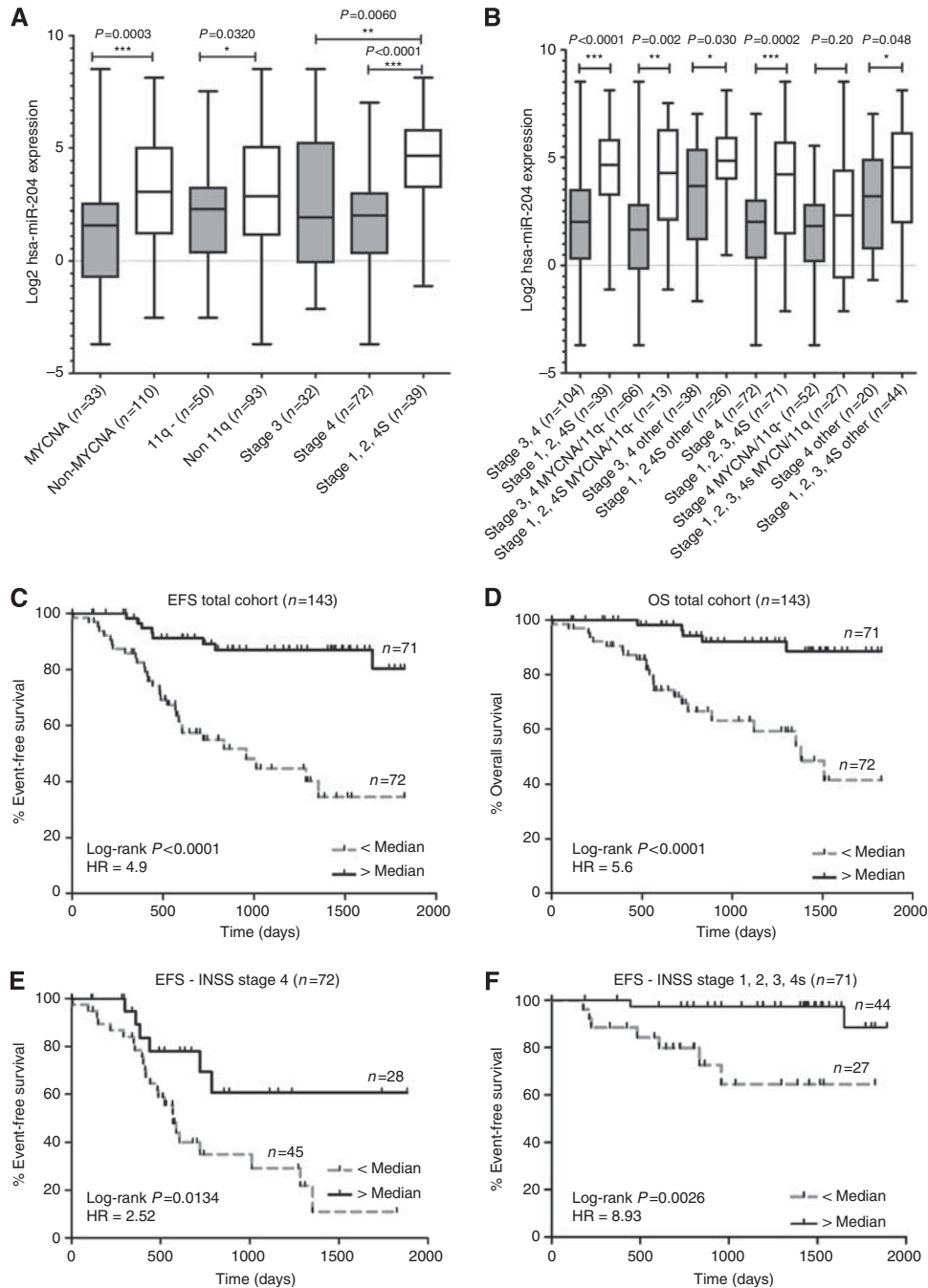
Figure 1 miR-204 expression is significantly associated with neuroblastoma disease subtypes and patient survival. (**A**) Box and whiskers plots represent the expression of miR-204 in a cohort of 143 primary neuroblastoma tumours. Samples are grouped according to known risk factors and miR-204 expression is compared between *MYCN* amplified (MYCNA) *vs* non-*MYCNA*, 11 LOH *vs* non-11q LOH, and INSS stages. Statistical differences in median 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.

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).

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

Table 1 Univariate (Kaplan–Meier) and multivariate (Cox proportional hazard regression) analysis of event-free survival in 143 neuroblastoma patients

| | | Univariate[a] | | | Multivariate[b] | | | | | |
| | | | | | Entered | | | Stepwise[c] | | |
Variable	n	HR	95% CI	EFS P	HR	95% CI	EFS P	HR[c]	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, 4S	71	I			I			I		
Stage 3, 4	104	22.5	11.7–43.1	<0.0001	6.49	0.8–52.6	0.0812	—	—	—
Stage 1, 2, 4S	39	I			I					
Age										
⩾1 year	123	2.42	1.1–5.4	0.0331	2.69	0.6–11.7	0.1815	—	—	—
<1 year	20	I			I					
MYCN										
Amplified	33	3.7	1.7–8.1	0.0011	1.80	0.9–3.8	0.1187	—	—	—
Non amplified	110	I			I					
11q										
Deletion	50	2.25	1.3–4.4	0.0154	1.28	0.6–2.7	0.5132	—	—	—
No deletion	93	I			I					
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	I			I			I		

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

survival time, leaving only INSS stage 4 *vs* stages 1, 2, 3, 4S and >/< median miR-204 expression as significant contributing variables (Table 1). In univariate survival analysis of stage-stratified patients, greater than median expression of miR-204 was significantly associated with improved EFS and OS in high-risk INSS stage 4 disease (EFS HR 2.53, 5-year EFS 11% *vs* 61%; OS HR 2.46, 5-year OS 10% *vs* 47%), and in stage 1, 2, 3, 4S disease (HR 8.9, 5-year EFS 64.5% *vs* 88.5%; OS HR 18, 5-year OS 79% *vs* 100%), indicating a degree of independence of miR-204 expression in determining time to relapse (Figure 1E and D).

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 (Supplementary Figure 2A). Transfection of miR-204 mimics into the NB1691[luc] and SK-N-AS[luc] cells before their implantation into the retroperitoneal space of SCID mice also had no impact on tumour establishment or growth *in vivo* (Supplementary Figure 2B).

In order to elucidate how higher miR-204 might contribute to improved patient survival, we assessed the effect of miR-204 overexpression on the response of two *MYCN*-amplified cell lines (Kelly and NB1691) to the chemotherapy drug cisplatin (CDDP), commonly used in the treatment of neuroblastoma. Kelly and NB1691 cells were transfected with miR-204 mimics and exposed to concentrations of CDDP ranging from 0 to 50 μg ml^{-1}. The survival of miR-204-transfected cells relative to the negative control, as determined by MTS assays, was signifi-

cantly lower at concentrations of 1 and 5 μg ml^{-1} CDDP in Kelly cells (Figure 2A), and at 5 μg ml^{-1} CDDP in NB1691 cells (Figure 2B). These observations were confirmed in further independent experiments using 5 μg ml^{-1} CDDP treatment (mean differences in cell viability; Kelly 8.94 ± 2.3% *P* < 0.001, NB1691 8.2 ± 2.6% *P* < 0.01) (Figure 2C and D).

Through *in silico* analysis (using the algorithms PicTar, TargetScan, and miRanda), we examined all computationally predicted target genes of miR-204. There was 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 apoptosis 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
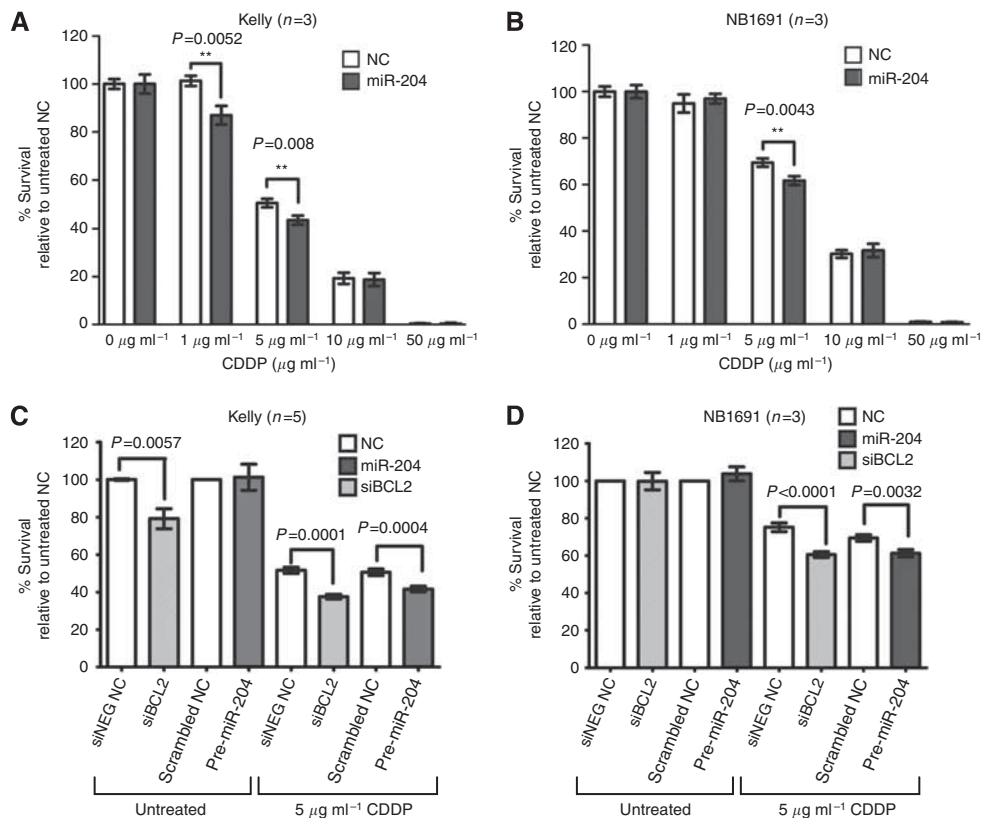
Figure 2 Ectopic expression of miR-204 in neuroblastoma cell lines increases sensitivity to cisplatin (CDDP) *in vitro*. (**A, B**) Neuroblastoma *MYCN*-amplified cell lines Kelly and NB1691 were transfected with miR-204 mimics or scrambled negative control (NC) oligonucleotides. At 48 h post transfection, 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 $\pm$ 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.

caspase activity translated to increased apoptotic rates in Kelly cells. Following treatment with 5 μg ml^{-1} CDDP, significantly increased levels of early apoptosis (Annexin V +/PI −) and late (Annexin V +/PI +) apoptosis were observed in miR-204 over-expressing cells, and in siBCL2 transfected cells, relative to the negative controls at 72 h (Figure 3B and C). Apoptosis levels were further enhanced at 96 h post transfection with 5 μg ml^{-1} CDDP treatment (Supplementary Figure 5). We conclude that miR-204, through enhanced apoptosis, significantly increases sensitivity of neuroblastoma cells to cisplatin *in vitro*.

To investigate if overexpression of miR-204 affected apoptosis of cells to chemotherapy challenge other than CDDP, we exposed cells to the topoisomerase II inhibitor etoposide (VP-16), also commonly used in the treatment of neuroblastoma. Following treatment with 10 μg ml^{-1} VP-16, modest but significantly increased levels of apoptosis (Annexin V positive: $33 \pm 4\%$ *vs* $23 \pm 2.4\%$ $P = 0.045$) were observed in miR-204 overexpressing Kelly cells, relative to the negative controls at 96 h (Supplementary Figure 6).

## 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
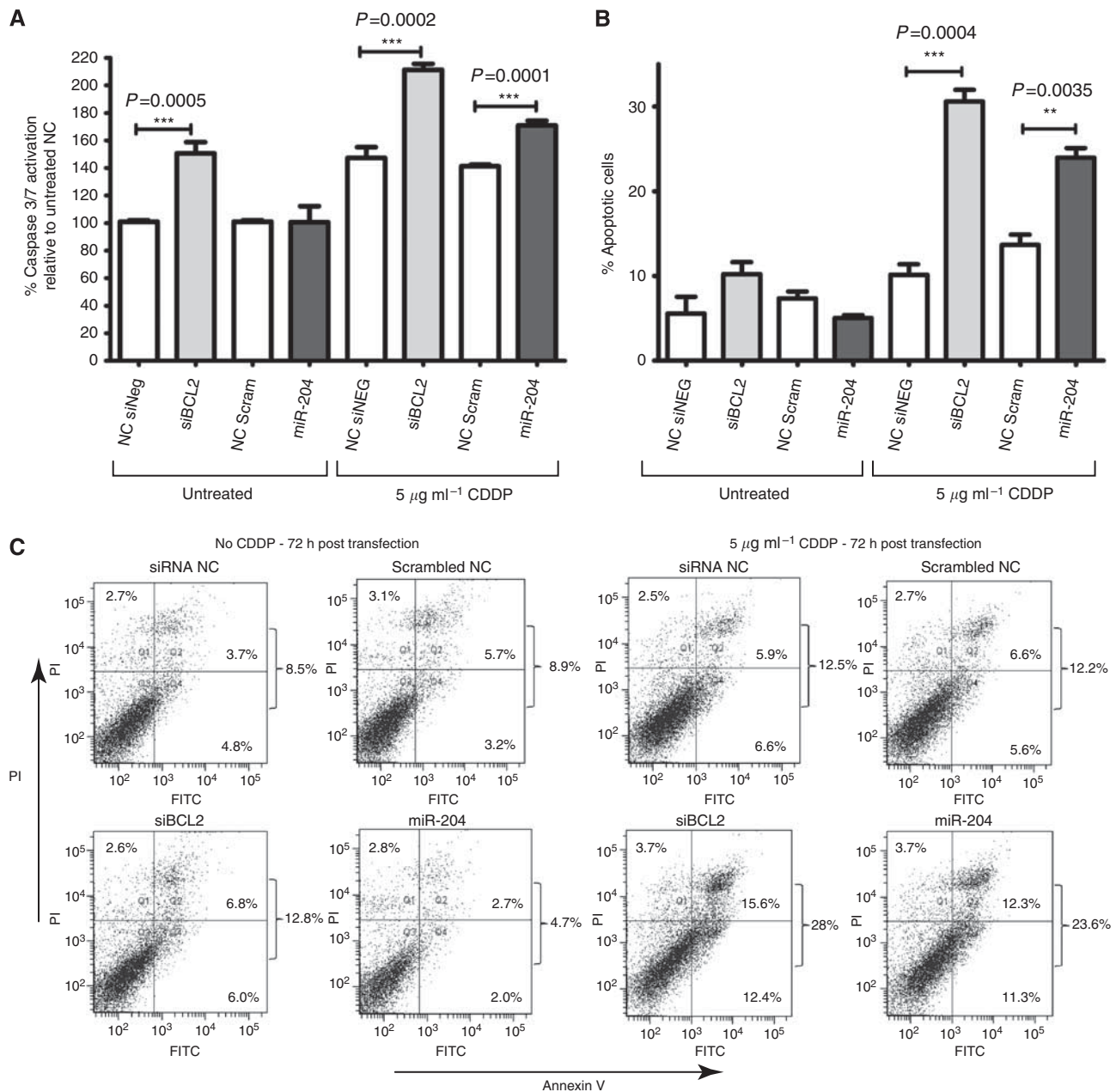
Figure 3 miR-204 increases apoptotic levels of neuroblastoma cells following treatment with CDDP. Kelly cells were transfected with siRNA targeting *BCL2* or siRNA negative control oligonucleotide (siNEG NC), or miR-204 mimics or scrambled negative control (scrambled NC) oligonucleotides. At 48 h post transfection, cells were treated for 24 h with or without 5 μg ml^{-1} CDDP. Graphed data are mean values $\pm$ 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 si*BCL2*/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.

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
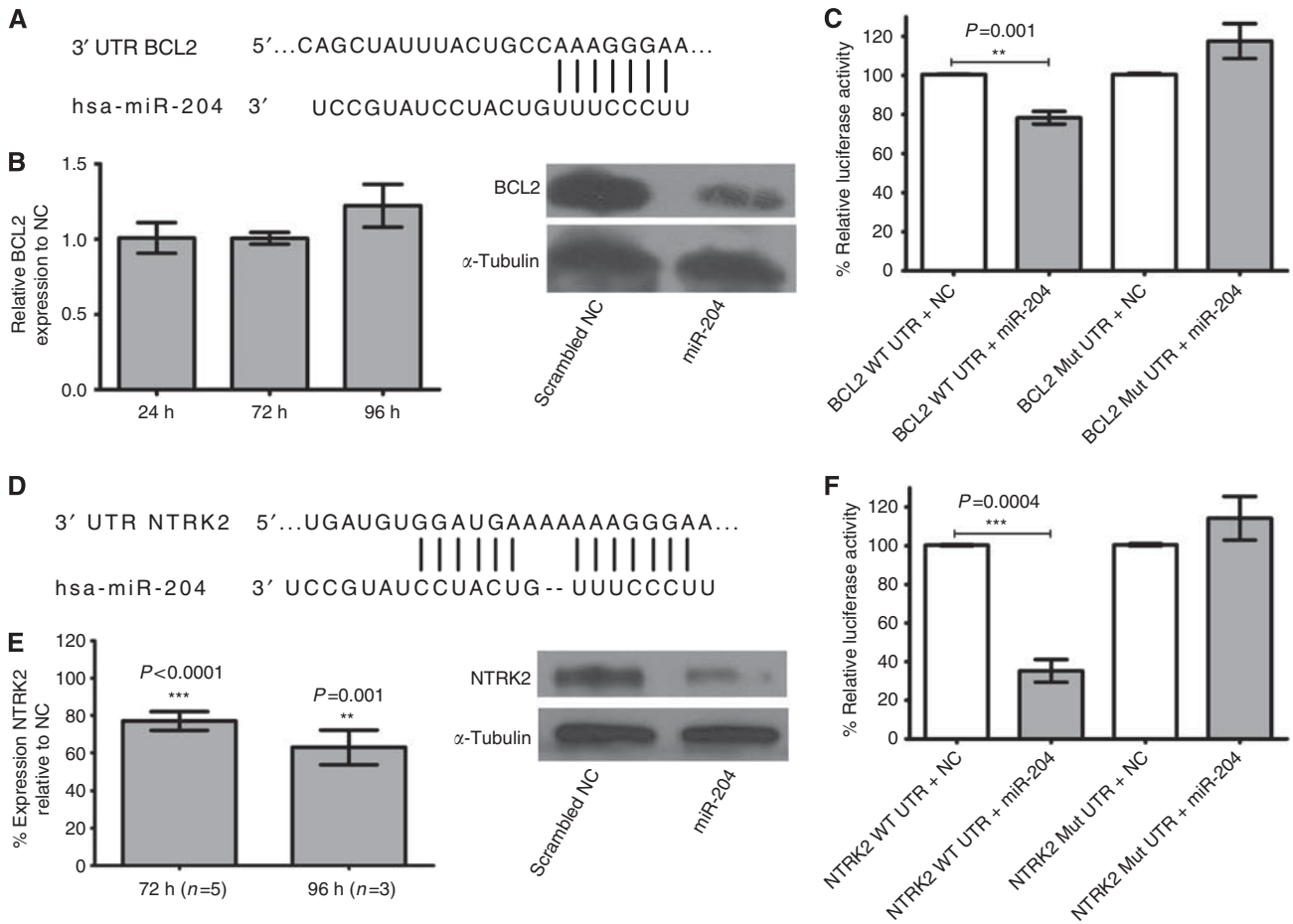
Figure 4 *BCL2* and *NTRK2* are direct targets of miR-204. Kelly cells were transfected with miR-204 mimics or scrambled negative control (scrambled NC) oligonucleotides. Graphed data are mean values ± s.e.m. of at least three independent experiments. (**A**) Conserved 8-mer seed match with hsa-miR-204 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; Mikhaylova *et al*, 2012). In the case of endometrial cancer, miR-204 was determined to regulate cell migration and invasion

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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) demonstated 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, Chen *et al* had 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 (Dole *et al*, 1994). In agreement with these observations, we showed that siRNA knockdown of *BCL2* in neuroblastoma cells resulted in a significant increase in apoptosis levels in response to cisplatin and etoposide treatment. Similarly, modest yet statistically significant increased apoptosis levels were observed in cells overexpressing miR-204 following cisplatin or etoposide treatment. That we also demonstrated that miR-204 directly targets the 3′ UTR of *BCL2*, and effectively reduces BCL2 protein levels in neuroblastoma cell lines, lends further credibility to our hypothesis that miR-204 represents a significant determinant of response to therapy in neuroblastoma. We suggest that the higher apoptosis levels induced by siRNA knockdown of BCL2 as opposed to miR-204-induced apoptosis levels is due in part to more effective targeting of BCL2 by siRNA, as multiple target sites might be competing for miR-204 binding. Lamers *et al* (2012) recently described that *BCL2* expression is highly upregulated in neuroblastoma tumours compared with normal controls and other cancers, but that these high levels of *BCL2* are not observed in a majority of neuroblastoma cell lines, and knockdown of BCL2 in low-expressing cell lines results in subtle or little effect on apoptosis. This is consistent with our observation that in the absence of cisplatin, siRNA knockdown of BCL2 had no significant effect on NB1691 cell lines and only a modest increase in apoptosis levels in Kelly cell lines. Although we determined that *BCL2* expression levels were readily detectable in our cell lines, it is worth considering that these levels may not be reflective of the high levels in patient tumours and that regulation of BCL2 protein *in vivo* may represent an even more dramatic phenotypic effect than we observed *in vitro*.

NTRK2 is also a well-established pro-survival oncogene in neuroblastoma, signalling the activation of the PI3K/AKT pathway, a significant mechanism of drug resistance in neuroblastoma (Bender *et al*, 2011). *NTRK2* is preferentially expressed in the brain and peripheral nervous system and, similar to *BCL2*, is essential for normal neuronal cell survival during development. Both *NTRK2*, and its ligand, *BDNF* are expressed at high levels in clinically unfavourable neuroblastoma, promoting cell survival and treatment resistance (Nakagawara *et al*, 1994; Ho *et al*, 2002; Jaboin *et al*, 2003). Interestingly, the seed region for miR-204 occurs only in the 3′ UTR of the full-length variant of *NTRK2* (as opposed to truncated versions). We determined that miR-204 specifically targets this 3′ UTR of *NTRK2* and ectopic expression of miR-204 results in a significant reduction of full-length NTRK2 protein, providing evidence of another distinct role of miR-204 in response to therapy and further highlighting the importance of miR-204 expression in neuroblastoma.

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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