The role of the long non-coding RNA ANRIL in chemosensitivity in osteosarcoma and clinical outcomes

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OSTEOSARCOMA

Osteosarcoma is a primary skeletal malignancy that affects children, adolescents, and young adults. Neoadjuvant chemotherapy and surgical resection is the mainstay of therapy. Patients receive an initial 2 cycles of chemotherapy with cisplatin, doxorubicin, and methotrexate. Following this, patients undergo resection of the primary tumor and undergo an assessment of necrosis of their primary tumor. Patients complete therapy with 4 more cycles of cisplatin, doxorubicin, and methotrexate.
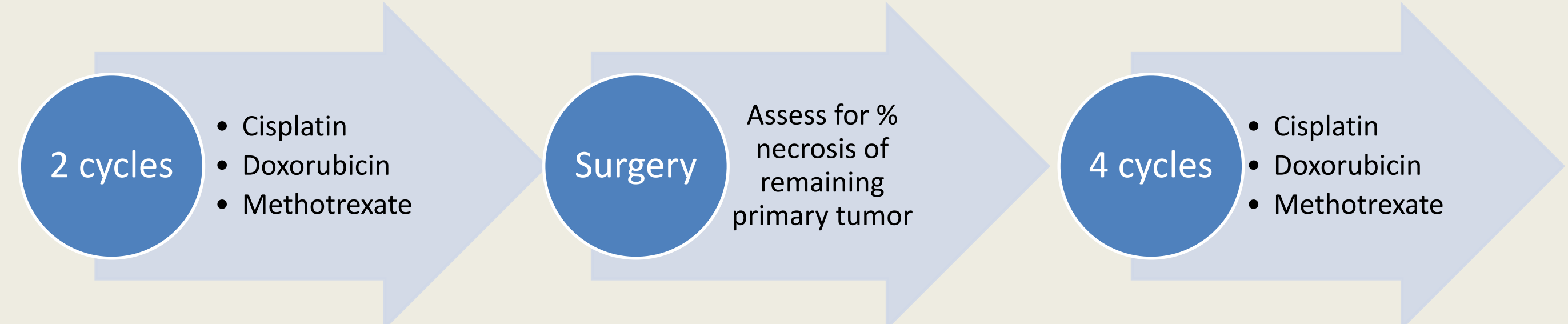

Figure 1: The current standard of care therapy for pediatric osteosarcoma in the United States

Prior to the introduction, widespread use, and standardization of the use of these chemotherapy agents, 5-year overall survival was poor at around 20%. The addition of neoadjuvant chemotherapy to the then back bone of surgical resection (or amputation) increased survival rates to 60-70%.

This initial increase in 5-year overall survival suggests that perhaps chemosensitivity, at least in part, can be viewed as a prognostic factor. Percent necrosis of the tumor cells is thought to predict outcome to some degree. Those whose primary tumor demonstrated >90% necrosis were deemed a good responder and those less than <90% were deemed a poor responder. Although the categorization of patients in responder status was initial promising, the clinical utility of this assignment is not as predictive as one would hope.

Only when the percent necrosis labels a patient a "poor responder," does it predict clinical outcome. However, this assessment and assignment of responder status unfortunately is preformed after 2 cycles of chemotherapy are completed. For the group of patients who do not demonstrate a good response after 2 cycles of chemotherapy, they may have been exposed to toxic chemotherapy that is not beneficial to them.
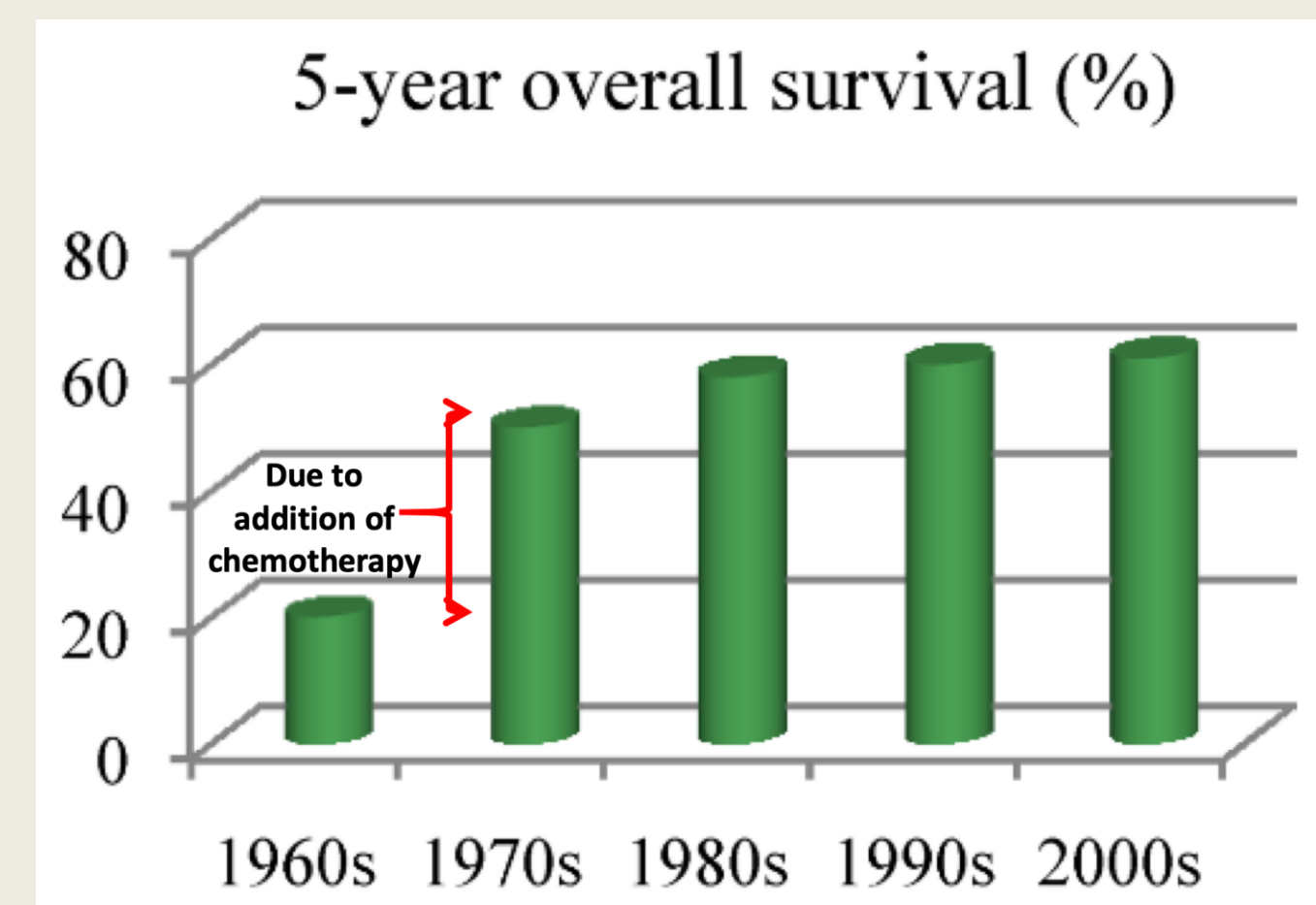

Figure 2: Change in 5-year overall survival (%)

Question: Is there a biological marker in osteosarcoma that can better predict chemosensitivity?

Long non-coding RNA (lncRNA)

Although there are known patient characteristics and protein coding gene expression/mutation statuses that can shed light on possible patient outcomes, there is a need for biomarkers to perhaps predict chemotherapeutic response. Cancer biomarkers form the protein-coding portion of the genome are known but are insufficient. The presence of Rb gene or p53 mutations are known osteosarcoma biomarkers but are poor markers to predict chemosensitivity. One area of recent interest is in long non-coding RNA or lncRNA.
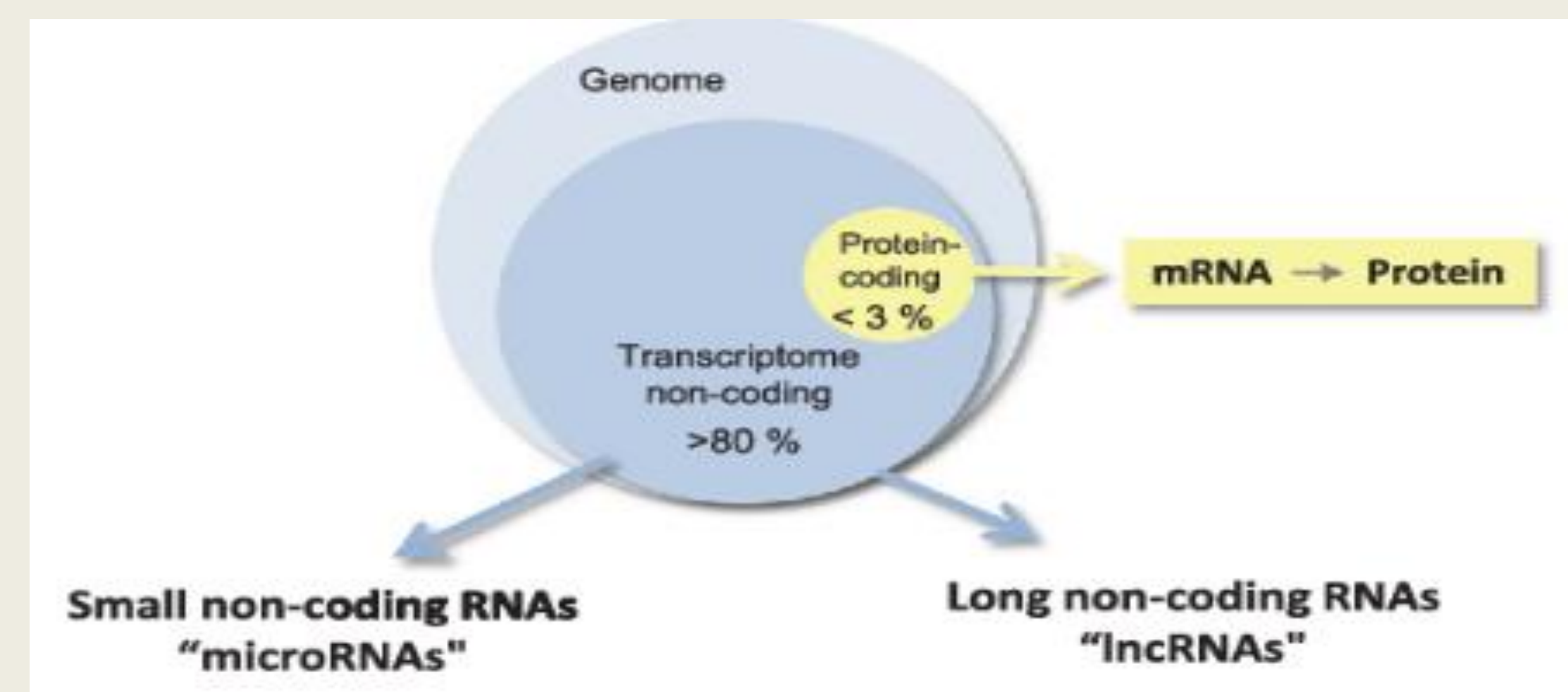

Figure 3: The portion of the human genome which contains non-coding transcriptome is greater than 80%. The protein-coding portion is less than 3%.

The lncRNA make up a majority of the transcriptome which is non-coding (60%) so there is an abundance of lncRNA. Their significance and contribution to cancer biology and pharmacology is emerging. Some identified lncRNA are known to manipulate local or global gene expression and are already implicated in the development of cancer metastases. However, the functionality is known for less than 1%.

HYPOTHESIS

Decreased ANRIL expression in an osteosarcoma cell line will lead to increased cisplatin and doxorubicin sensitivity and improved clinical outcomes.

METHODS

In Dr. R. Stephanie Huang's lab, there has been much work done in understanding the possible role of lncRNA in cancer biology. Utilizing high-throughput large-scale cancer cell lines (approximately 900 cancer cell lines, each with detailed RNA-seq data), we focused on 3 chemotherapies to demonstrate expression.

The lncRNA ANRIL demonstrated a strong association with drug sensitivity to cisplatin, doxorubicin, and methotrexate (**p-value = 0.0013, 0.0035, 0.0053**, respectively). The directionality is such that increasing ANRIL expression causes increased resistance to these agents.

ANRIL stands for *antisense noncoding RNA in the INK4 locus* and is anti-sense to a gene cluster which encodes 3 important tumor suppressor proteins which lead to epigenetic silencing and therefore functions as an oncogene.
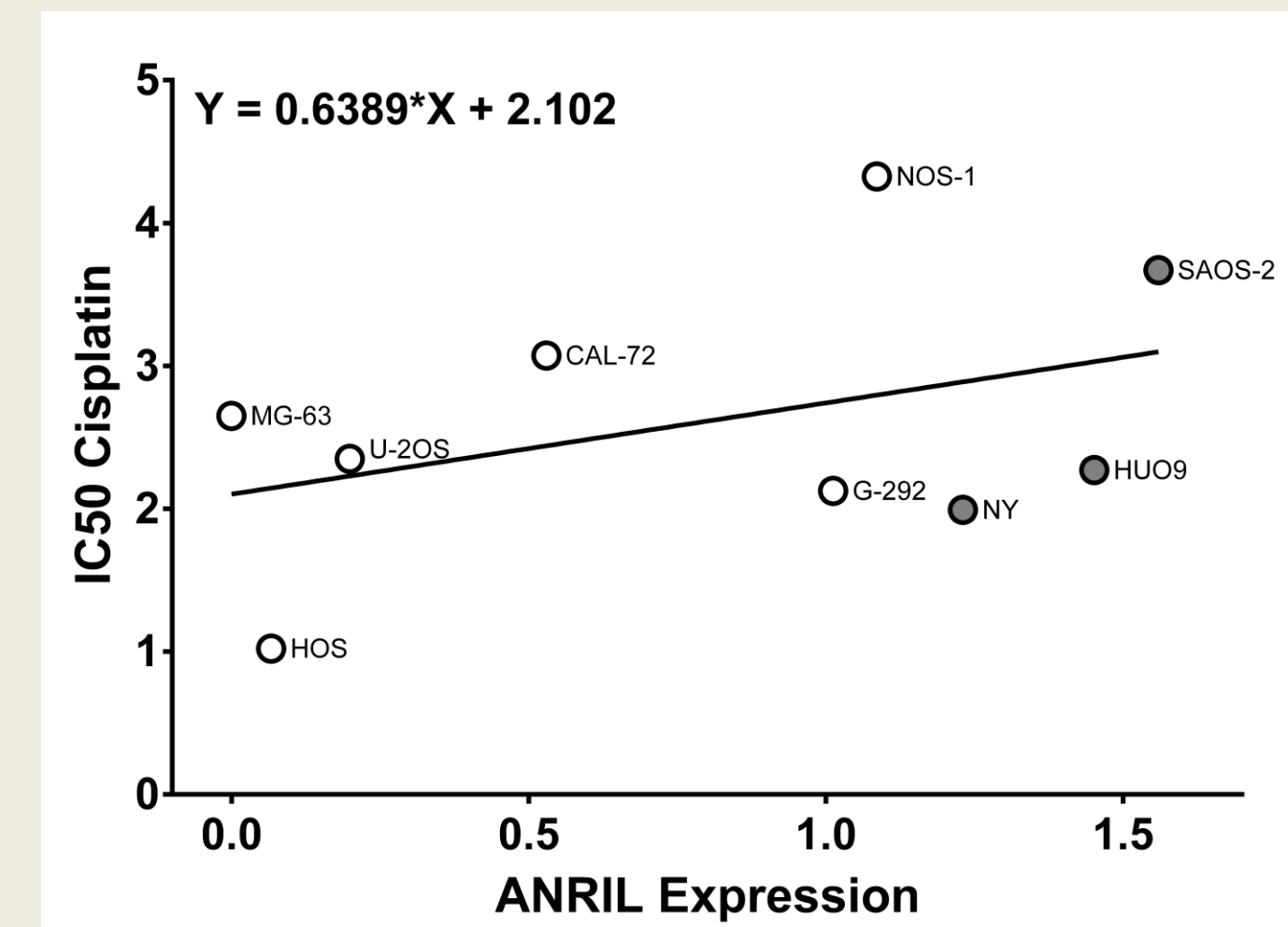

$Y = 0.6389*X + 2.102$

The figures to the left demonstrate the relationship between ANRIL expression here on the x-axis and the IC50 of cisplatin and the IC50 of doxorubicin across available osteosarcoma cell line data.

IC50 can be used as a measure of drug sensitivity such that as IC50 increases, the less sensitive a drug is measured to be.

In some cells, particularly SaOS2, increased ANRIL expression is associated with decreased sensitivity in cisplatin.

Figure 4: ANRIL expression versus the IC50 of cisplatin across osteosarcoma cell lines

$Y = 0.6109*X + 0.2138$

Likewise, in figure 5, for the cell line HUO9 as an example, increased ANRIL expression is associated with decreased sensitivity to doxorubicin.

For this work, since the intention was to knockdown ANRIL to assess for increasing sensitivity to cisplatin and eventually doxorubicin; we chose to focus on the osteosarcoma cell line SaOS2 with the greatest ANRIL expression.
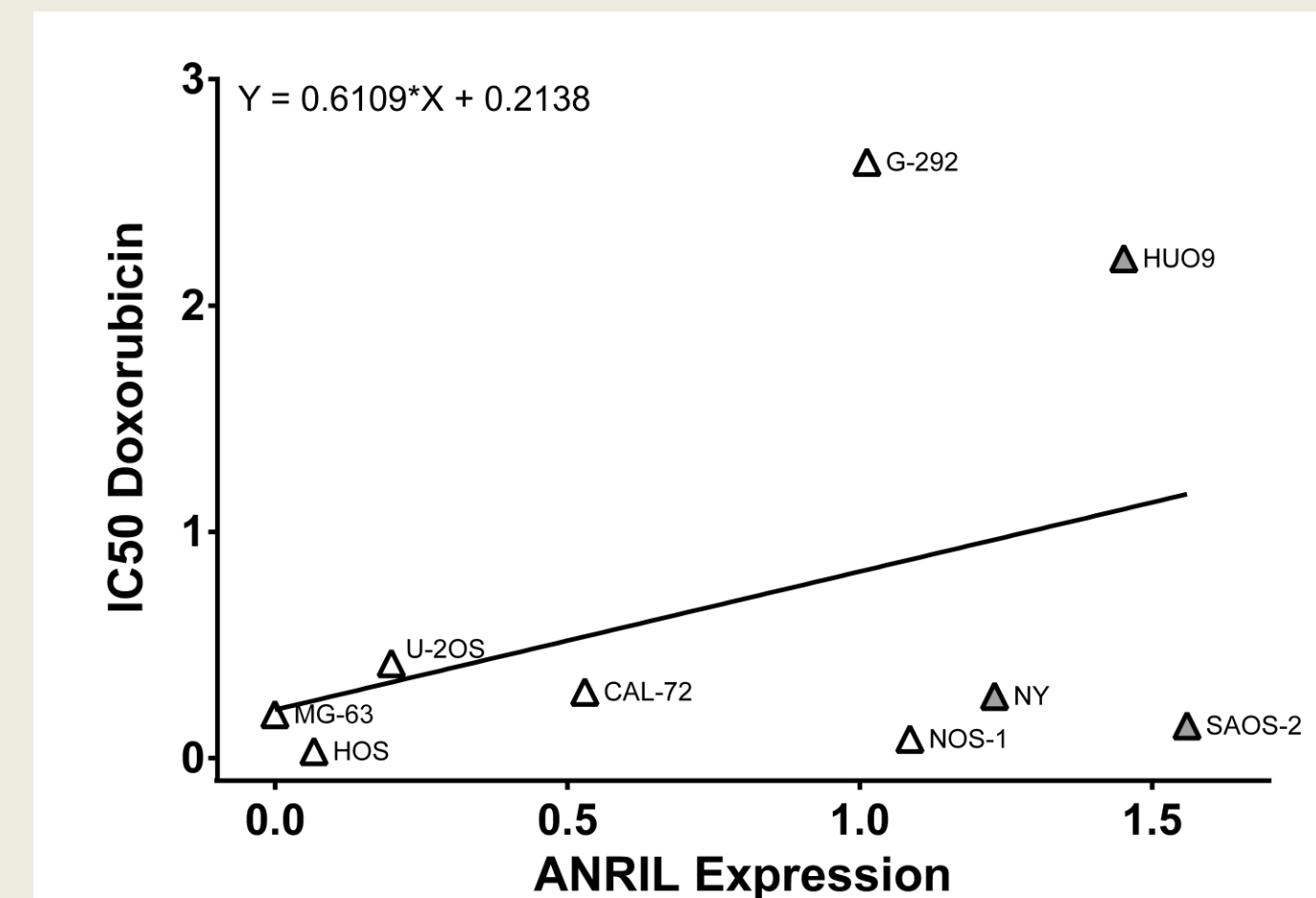
Figure 5: ANRIL expression versus the IC50 of doxorubicin across osteosarcoma cell lines
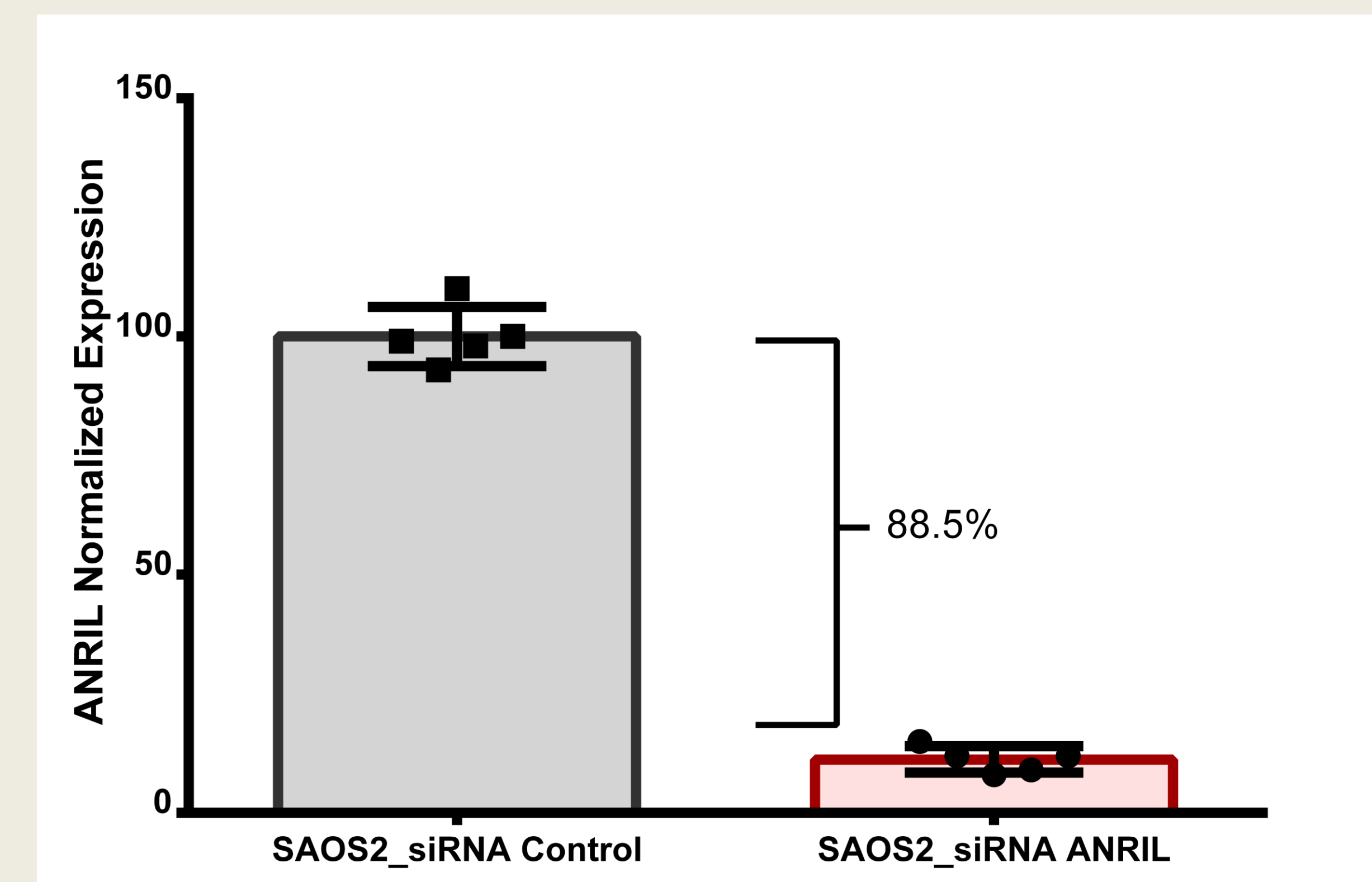

Figure 6: siRNA knockdown was preformed at 88.5% efficiency when comparing the siRNA conditions with normalized ANRIL expression
The osteosarcoma cell line SaOS2 was established in our lab and ANRIL expression was knocked down via siRNA transfection.

RESULTS

The SaOS2 cells were exposed to increasing concentrations of cisplatin or doxorubicin. Cellular sensitivity to these drugs was compared between ANRIL siRNA and scramble control at 24, 48, and 72 hours.
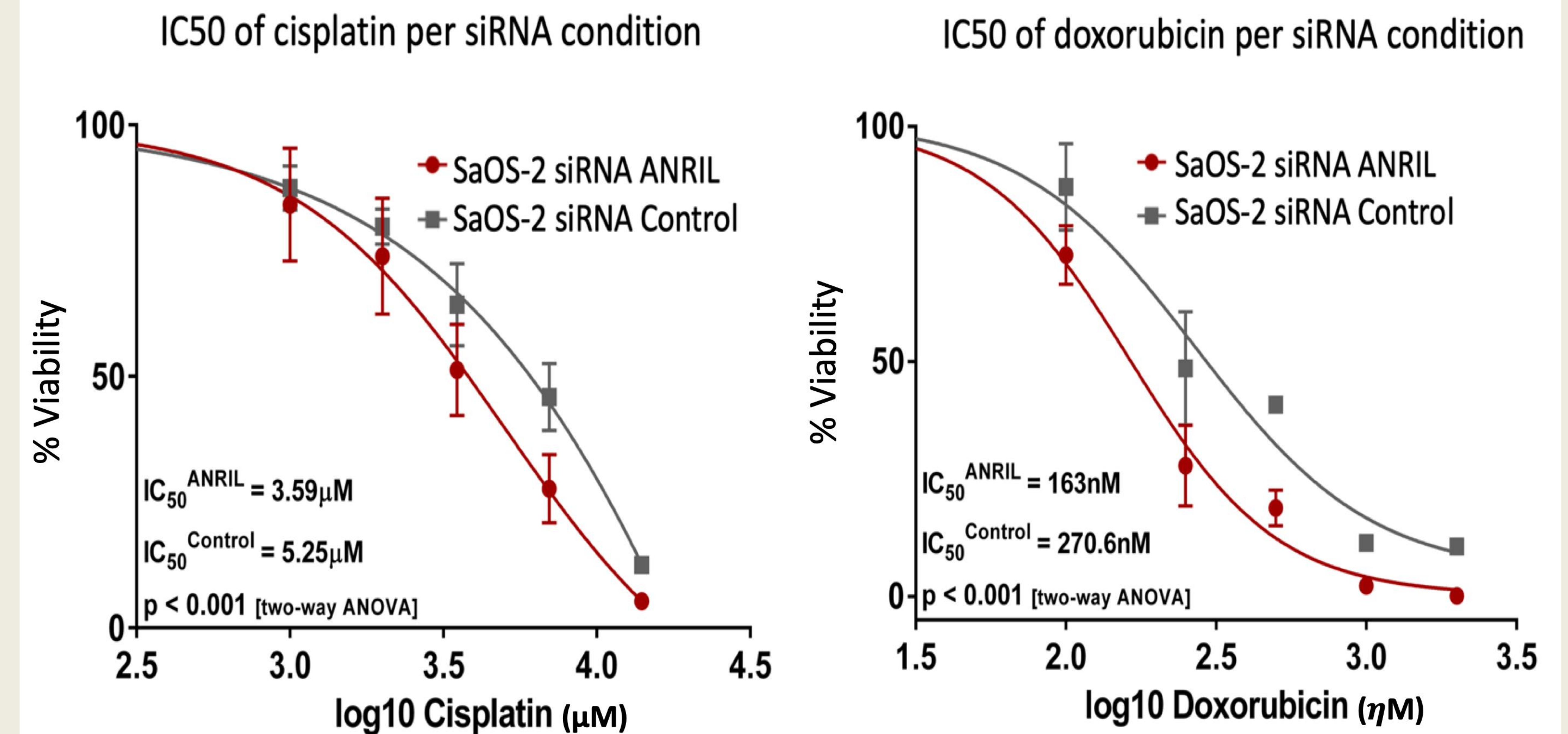

$IC_{50}^{ANRIL} = 3.59\mu M$
$IC_{50}^{Control} = 5.25\mu M$
$p < 0.001$ [two-way ANOVA]

$IC_{50}^{ANRIL} = 163nM$
$IC_{50}^{Control} = 270.6nM$
$p < 0.001$ [two-way ANOVA]

Figure 7: A. Decreased IC50 of cisplatin in the siRNA ANRIL knockdown condition, p<0.001 **B.** Decreased IC50 of doxorubicin in the siRNA ANRIL knockdown condition, p<0.001

Figure 7A demonstrates a statistically significant decrease in the IC50 of cisplatin in the siRNA knockdown condition as compared to the scramble control. There is a decline in the IC50 from 5.25 to 3.59 after ANRIL knockdown indicating that the SaOS2 osteosarcoma cells become more sensitive to cisplatin. Figure 7B illustrates the effect with doxorubicin and demonstrates a statistically significant decrease in the IC50 of doxorubicin in the siRNA knockdown condition as compared to the scramble control. There is a decline in the IC50 from 270.6 to 163 after ANRIL knockdown indicating that the SaOS2 osteosarcoma cells become more sensitive to doxorubicin. In summary, this suggests that knockdown of ANRIL in osteosarcoma cells leads to increased sensitivity to cisplatin and doxorubicin.

CLINICAL CORRELATION

Knowing that the knockdown of ANRIL leads to increased sensitivity of osteosarcoma cells to cisplatin and doxorubicin was important but we wanted to know if this increased sensitivity translated to improved clinical outcomes. We utilized two independent clinical datasets: TARGET (Therapeutically Applicable Research To Generate Effective Treatments) and BOOST (Biology of Osteosarcoma Study Team).

TARGET contained 121 tumor samples with RNA seq data available compared to just 35 from BOOST. The following box plots represent data from the TARGET database.
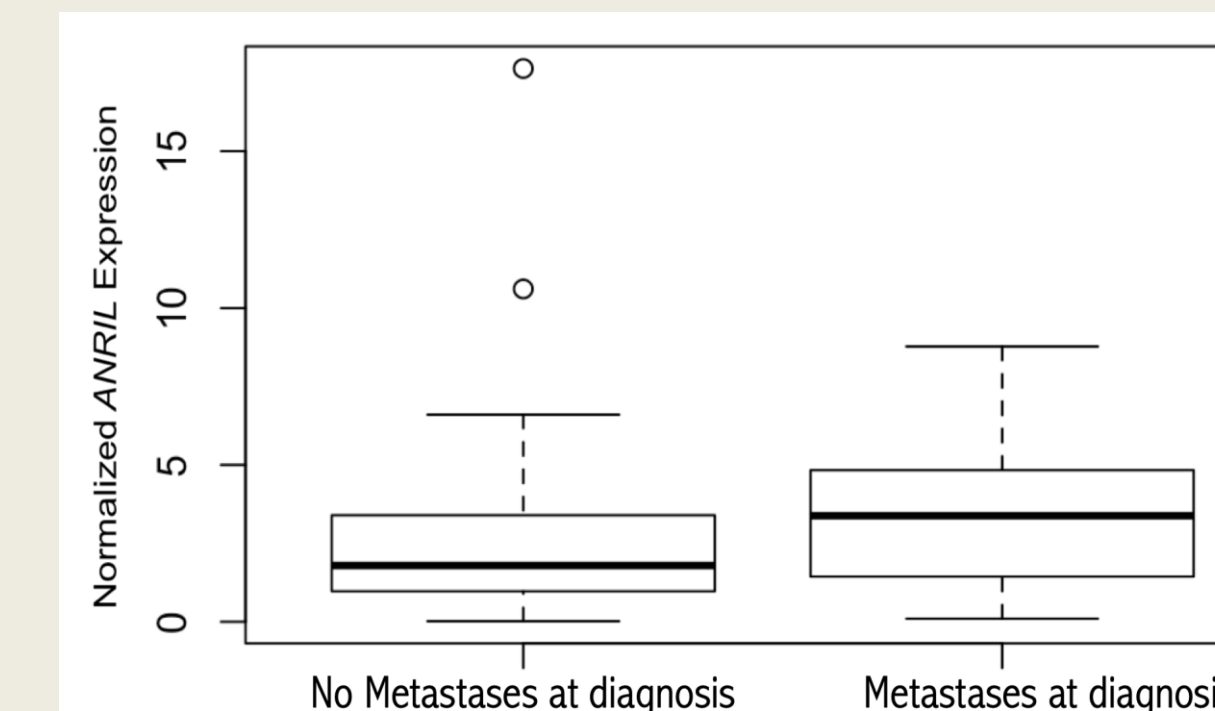

Figure 8: Difference in ANRIL expression for patients with metastases at diagnosis and those without metastases at diagnosis with a p-value for the association between metastases at diagnosis and ANRIL expression of 0.013.

Figure 9: Difference in ANRIL expression for patients who were alive and who were dead with a p-value for the association between death and ANRIL expression of 0.004.

This may suggest that increased ANRIL expression and thus increased resistance to cisplatin and doxorubicin may predict clinical outcomes.
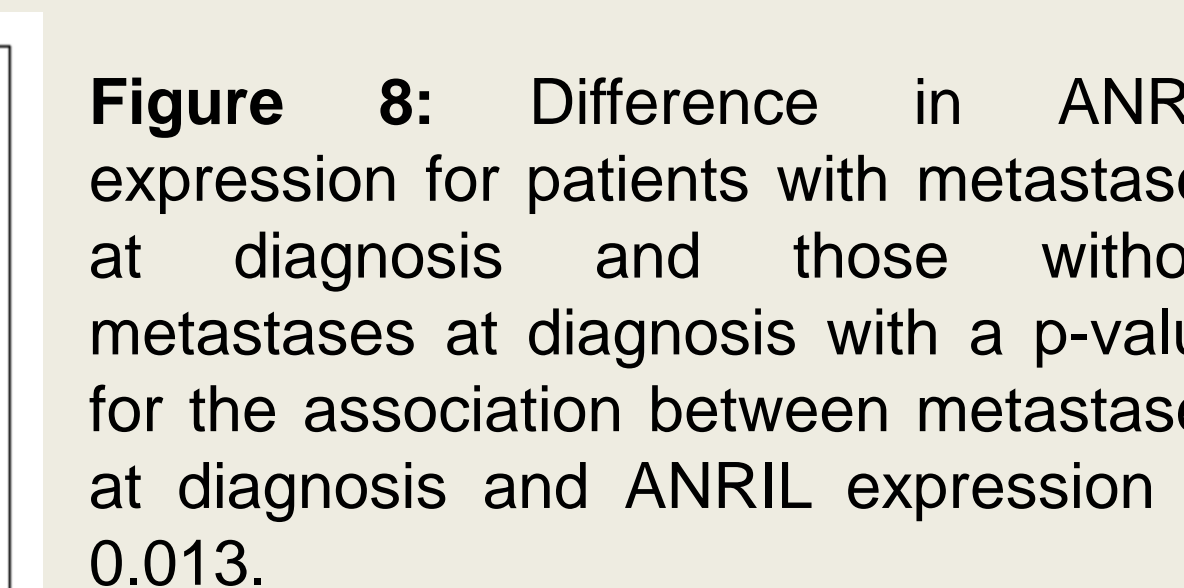
CONCLUSIONS

Knocking down the lncRNA ANRIL, thereby decreasing its expression, increases the sensitivity of osteosarcoma cells to cisplatin and doxorubicin in an osteosarcoma cell line. High ANRIL expression is associated with increased death and metastases at diagnosis in a statistically significant manner in a clinical dataset. Therefore, ANRIL may serve as a biomarker in predicting impending chemoresistance in patients with osteosarcoma.

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ACKNOWLEDGEMENTS