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Digital quantitation of HCC-associated stem cell markers and protein quality control factors using tissue arrays of human liver sections

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ABSTRACT

The most common type of liver cancer, hepatocellular carcinoma (HCC), affects over 500,000 people in the world. In the present study, liver tumor resections were used to prepare tissue arrays to examine the intensity of fluorescence of IHC stained stem cell markers in liver tissue from malignant HCC tumors and accompanying surrounding non-tumor liver. We hypothesized that a correlation exists between the fluorescence intensity of IHC stained HCC and surrounding non-tumor liver compared to liver tissue from a completely normal liver. 120 liver resection specimens (including four normal controls) were placed on a single slide to make a tissue array. They were examined by digitally quantifying the intensity of fluorescence using immuno-histochemically stained stem cell markers and protein quality control proteins. The stem cell markers were OCT3/4, Nanog, CD133, pEZH2, CD49F and SOX2. The protein quality control proteins were FAT10, UBA-6 and ubiquitin. The data collected was used to compare normal liver tissue with HCCs and parent liver tissue resected surgically using antibodies to stem cell markers and quality control protein markers. The measurements of the stem cell marker CD133 indicated an increase of fluorescence intensity for both the parent liver tissue and the HCC liver tissues. The other stem cell markers changed as follows: Nanog and OCT3/4 were decreased in both the HCCs and the parent livers; PEZH2 was reduced in the HCCs; SOX2 was increased in the parent livers compared to the controls; and CD49f was decreased in HCCs only. Protein quality control markers FAT10 and ubiquitin were downregulated in both the HCCs and the adjacent non-tumor tissue compared to the controls. UBA6 was increased in both the HCCs and the parent livers, and the levels were higher in the HCCs compared to the parent livers.

1. Introduction

Patients who develop hepatocellular carcinoma (HCC), account for 90% of all primary liver tumors and have an estimated survival rate of 6 to 20 months without intervention.

According to previous work on stem cells in human liver diseases, the presence of stem cells is found in both HCCs and in the surrounding diseased livers (Lingala et al., 2010; Oliva et al., 2010). In the present study, stem cell marker proteins, protein quality control proteins and tumor suppressor proteins in HCCs' parent livers, and normal control livers were semi-quantified by measuring fluorescent intensity of labeled antibodies using the immuno-histochemical staining method of digital morphometrics. The comparisons made using tissue arrays consisted of 120 livers present on a single slide. Three samples of each liver specimen were measured. The results were correlated with the results of previous studies where quantification of gene expression and immunofluorescent intensity was performed by PCR (French et al., 2012; Lui et al., 2014).

2. Materials and methods

A tissue microarray was generated from archived pathology cases at UCLA composed of either partial resections or hepatectomies. The array was constructed in the laboratory of Dr. Jiaoti Huang by Jill Squires at UCLA. All work was performed with appropriate institutional review board approvals. Liver tissue was stained for different markers (UBA-6, NANOG, FAT10, CD133, OCT3/4, ubiquitin, pEZH2, SOX2, CD49F). The slides of liver tissue arrays were examined using the NIS-Elements D 4.13.00 morphometric software and a Nikon Eclipse E400 fluorescence microscope. The liver tissue was viewed with a calibration of Plan Fluor 40× objective and, exposure at 800 ms.

Three samples from 120 resected livers (116 HCCs, 116 parent livers and 4 normal liver controls) were quantitated. The fluorescent intensity was measured using the intensity profile. The intensity profile creates a

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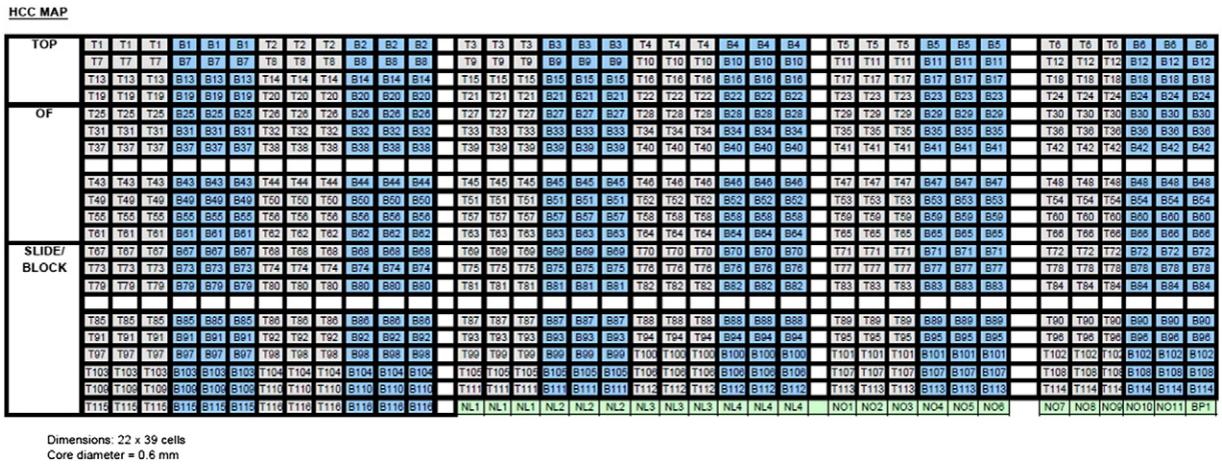


Fig. 1. HCC tissue array map. Map of tissue array composed of 120 liver resection specimens.

graph of sequential intensity changes and allows the measurement of numeric data by recording the intensity in the peaks on the graph. For each sample examined, one random snip was taken and 15 measurements were recorded from the intensity profile graph. The tissue array grid is shown in Fig. 1. A sample of the tissue samples is shown in Fig. 2.

2.1. Statistical analysis

SigmaStat software was used to establish the mean, SEM, P values, and one-way ANOVAs within the data recorded. SigmaPlot software was used to analyze the visual results of the research conducted.

3. Results

The protein quality control protein, UBA-6 demonstrated a marked increase of fluorescence intensity in the parent liver (B) and the HCC liver tumors (T) in comparison to the tissue from the normal livers (NL) (Fig. 3). In the case of the stem cell marker Nanog, the fluorescence intensity was markedly lower in the T and B liver samples in comparison to the NL liver samples (Fig. 4). NL liver samples showed a higher intensity than T and B liver samples measuring the intensity of the stem cell marker FAT10 (Fig. 5). OCT3/4 showed a decrease in fluorescence intensity in the T and B cells (Fig. 6). B samples were slightly more intensity fluorescent than + HCC samples. Ubiquitin (UB) displayed a lower fluorescence intensity than NL liver tissue in the T and B liver samples (Fig. 7). The HCC tissue showed a greater fluorescent intensity than the parent liver. With the DNA methylase pEZH2, tumor liver samples resulted in the lowest fluorescent intensity, compared with B tissue and control tissue (Fig. 8). SOX2 was increased in the parent tissue compared to the HCC tissue but not in the normal liver controls (Fig. 9). The stem cell marker, CD133 also showed an increase in the T and B liver samples in comparison to NL liver samples (Fig. 10). CD49F displayed a decrease in fluorescence intensity in the HCC tissues compared to the parent tissues and controls (Fig. 11). The level of fluorescent intensity was the greatest with FAT10 and CD49f and was the lowest in UBA6 and Nanog.

4. Discussion

FAT10 and ubiquitin were downregulated in the HCC samples and the parent livers compared to the normal control livers. This is consistent with a downregulation of the protein quality control FATylation pathway where misfolded proteins accumulate because the rate of their degradation is slowed due to the lack of the proteasome pathway of protein turnover (Lui et al., 2014). FAT10 was downregulated when measured by PCR in human HCC (data not shown).

UBA6 was upregulated in both HCC and parent liver tissue, HCC more than the parent liver. UBA6 is involved in FATylation. PEZH2

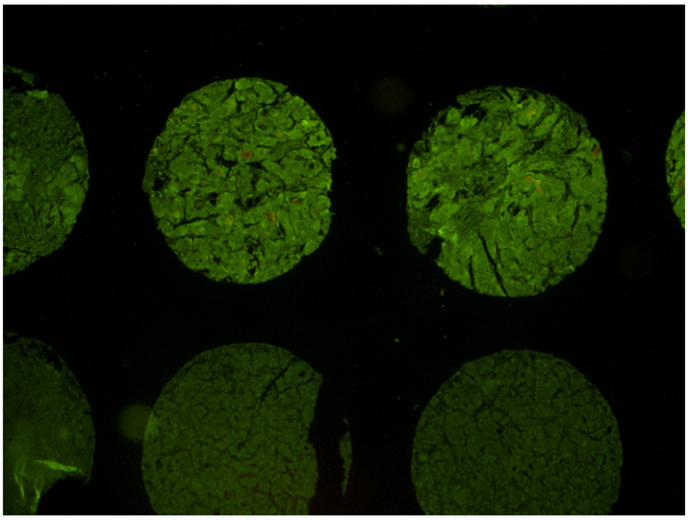


Fig. 2. Low power (4×) fluorescent image of tissue array stained for Nanog.

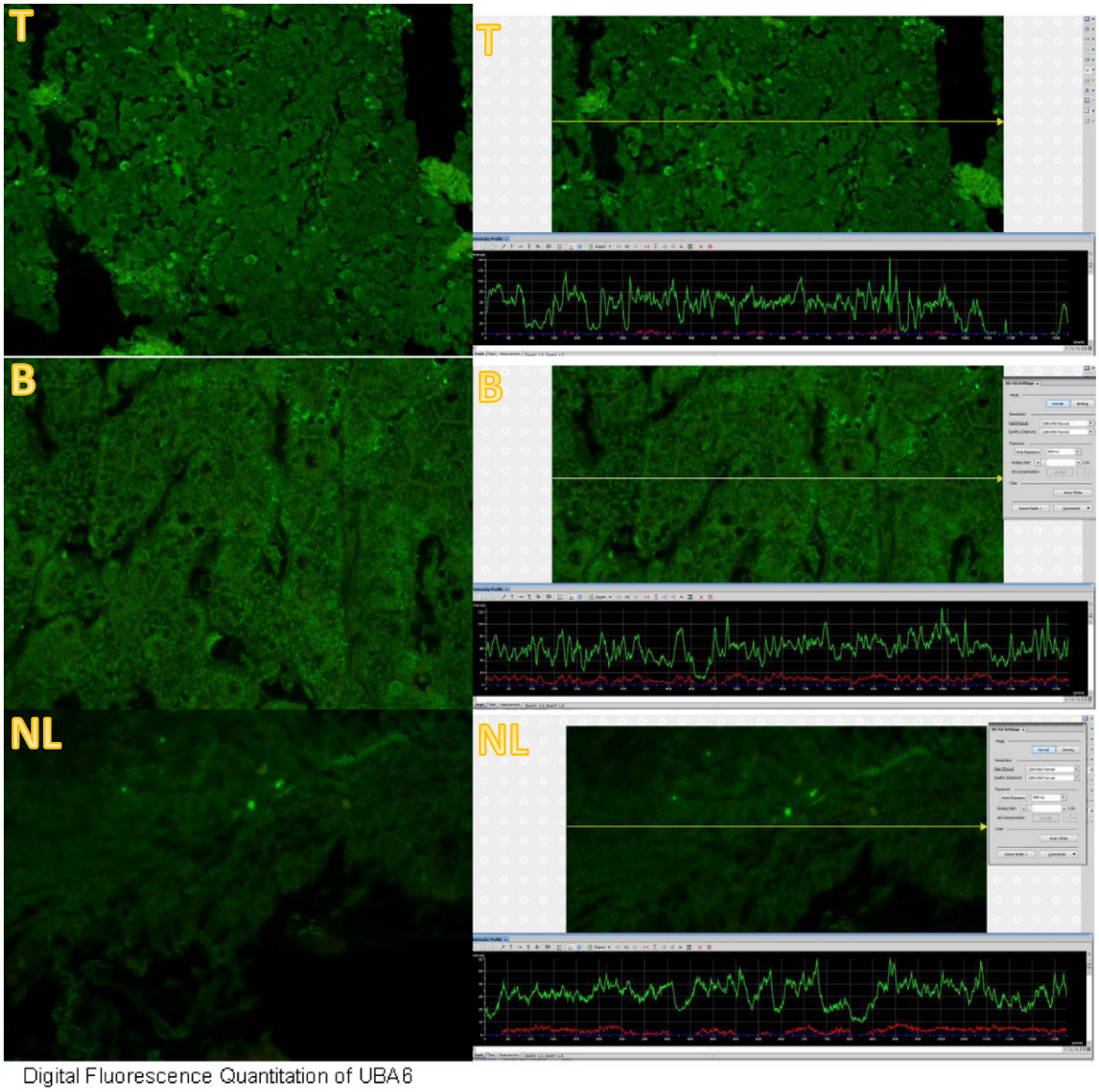
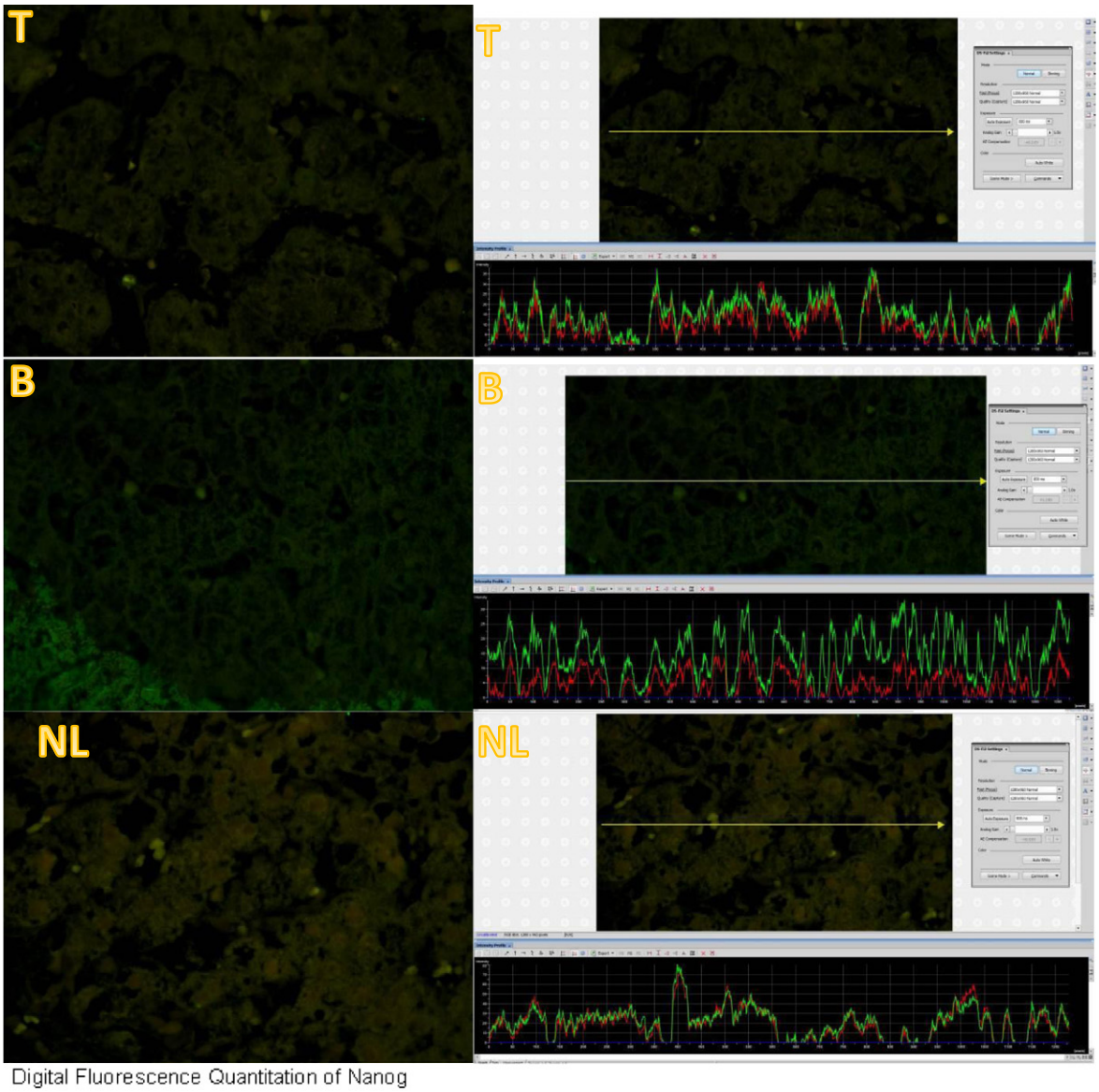


Fig. 3. UBA-6 was upregulated in T (tumor) and B (parent liver) resections compared to NL (normal liver). Intensity profiling of the stained tissue array under fluorescence microscopy (40× objective) demonstrated the greater presence of UBA-6, as indicated by the higher peaks in the T and B screen snips shown as compared to the NL intensity peaks. Compared to normal liver control resections, T and B exhibited an average higher intensity. The results are shown as Mean ± S.E.



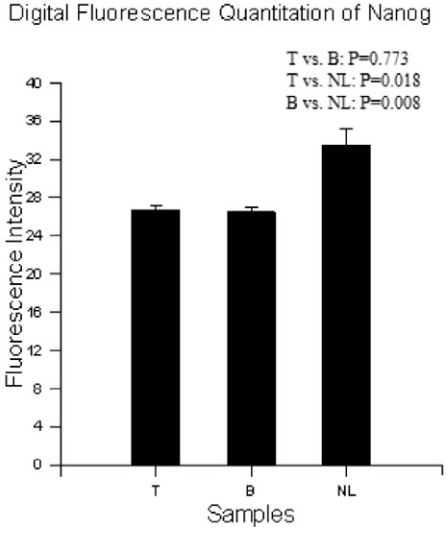


Fig. 4. The presence of Nanog decreased in T and B resections. Fluorescence intensity profiling of stained tissue array indicated lower intensity peaks in T and B resections compared to normal liver tissue. Compared to normal liver control resections, T and B exhibited an average lower fluorescence intensity; the results are displayed as Mean ± S.E.

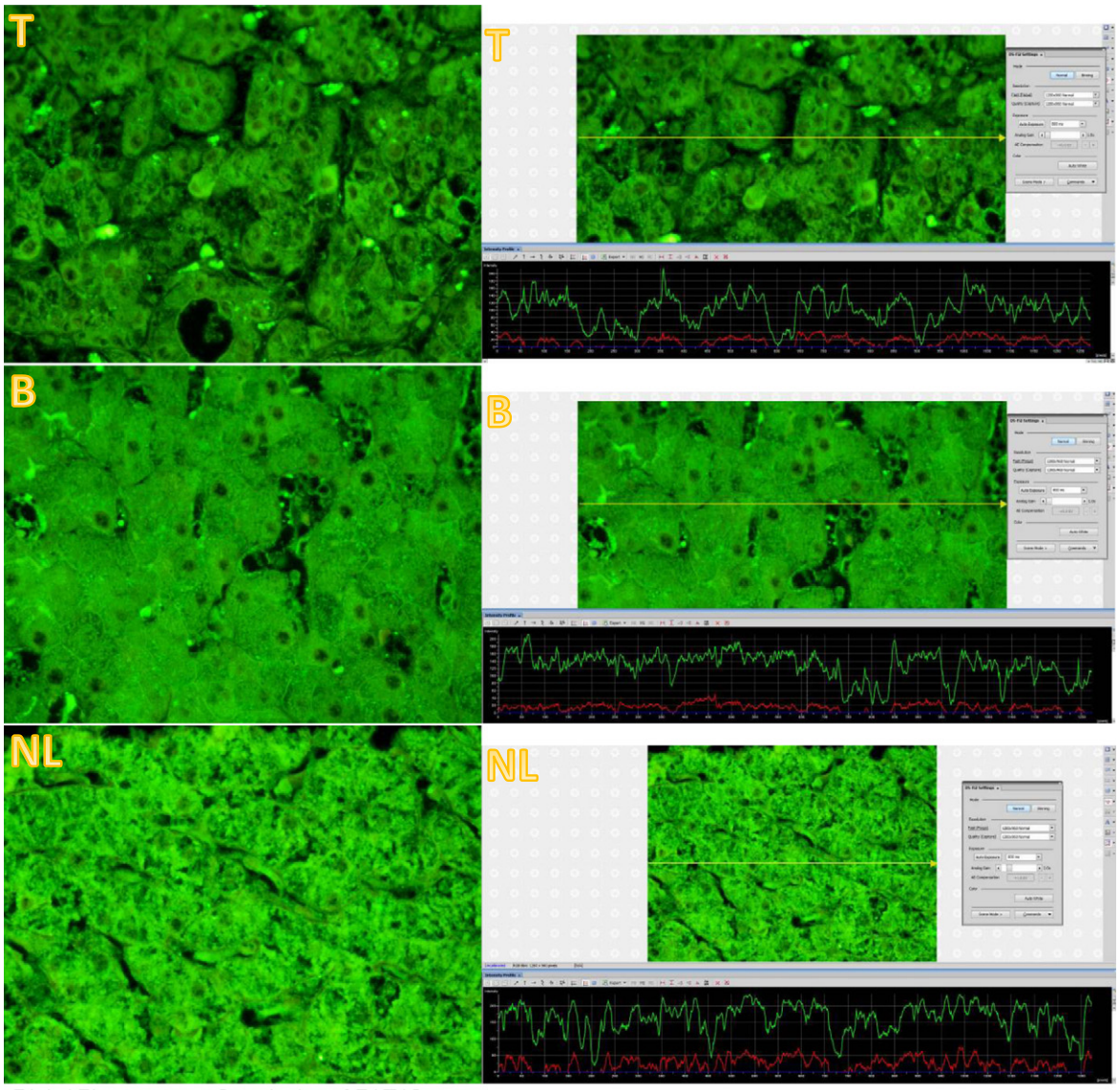


Fig. 5. FAT10 was downregulated in T and B resections. Fluorescence intensity profiling of the stained tissue array demonstrated a decrease in FAT10 in T and B resections, shown by the lower intensity peaks as compared to normal liver control resections. T and B showed an average lower intensity; the results are displayed as Mean ± S.E.

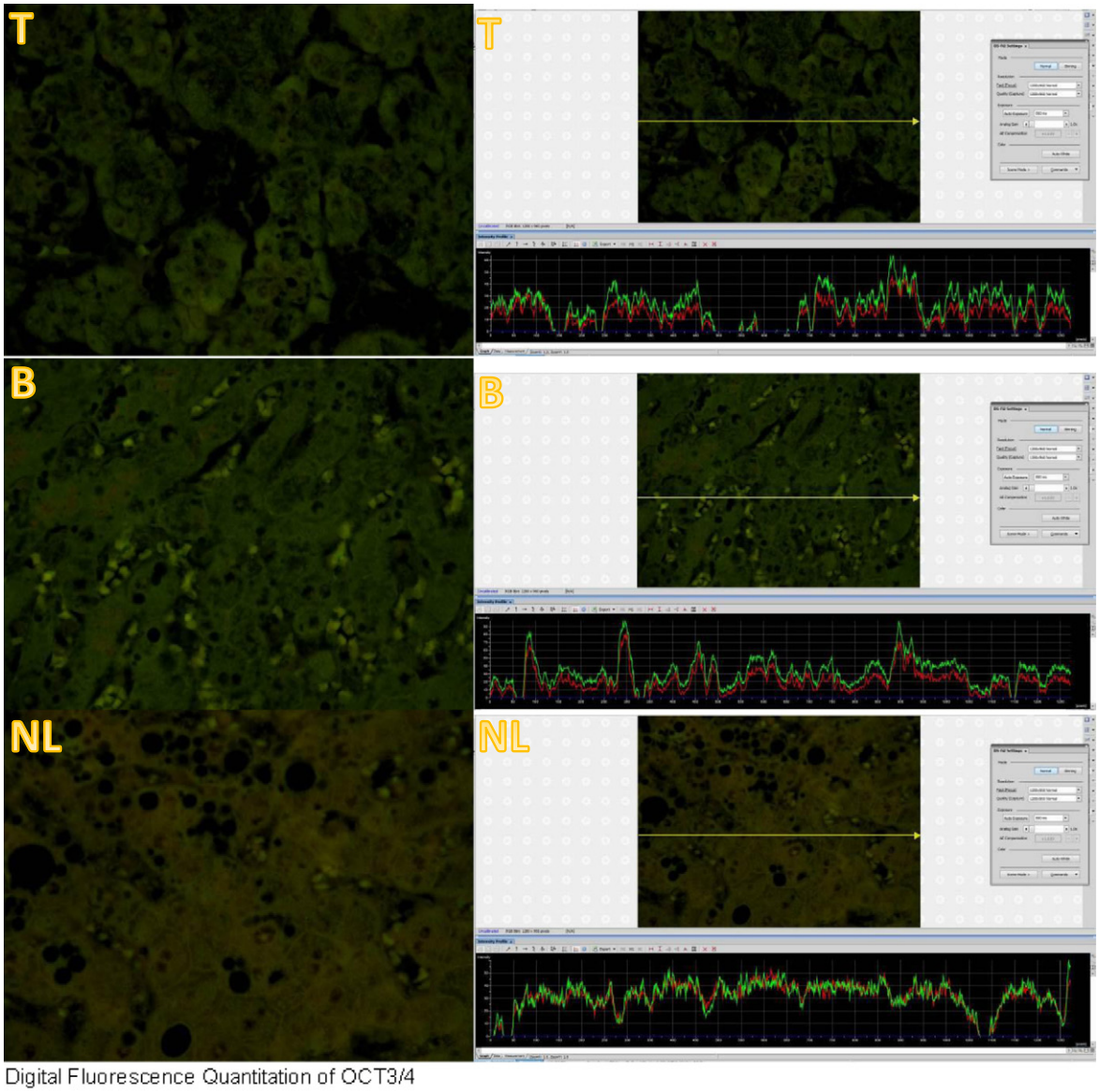


Fig. 6. OCT3/4 decreased in T and B resections. Fluorescence intensity profiling of the stained tissue array demonstrated lower fluorescence intensity than the normal liver resections. The results displayed (Mean ± S.E.,) exhibit lower intensity than the control resections.

decreased in the HCCs' tissue, which would explain the decrease in H3K27me3 of DNA methylation observed (Chen et al., 2010; Zeng et al., 2010).

Nanog is one of the primary markers that turn benign stem cells into cancer stem cells (TISC) (Machida et al., 2012). In the present study, Nanog expression was decreased in fluorescence intensity in both the T

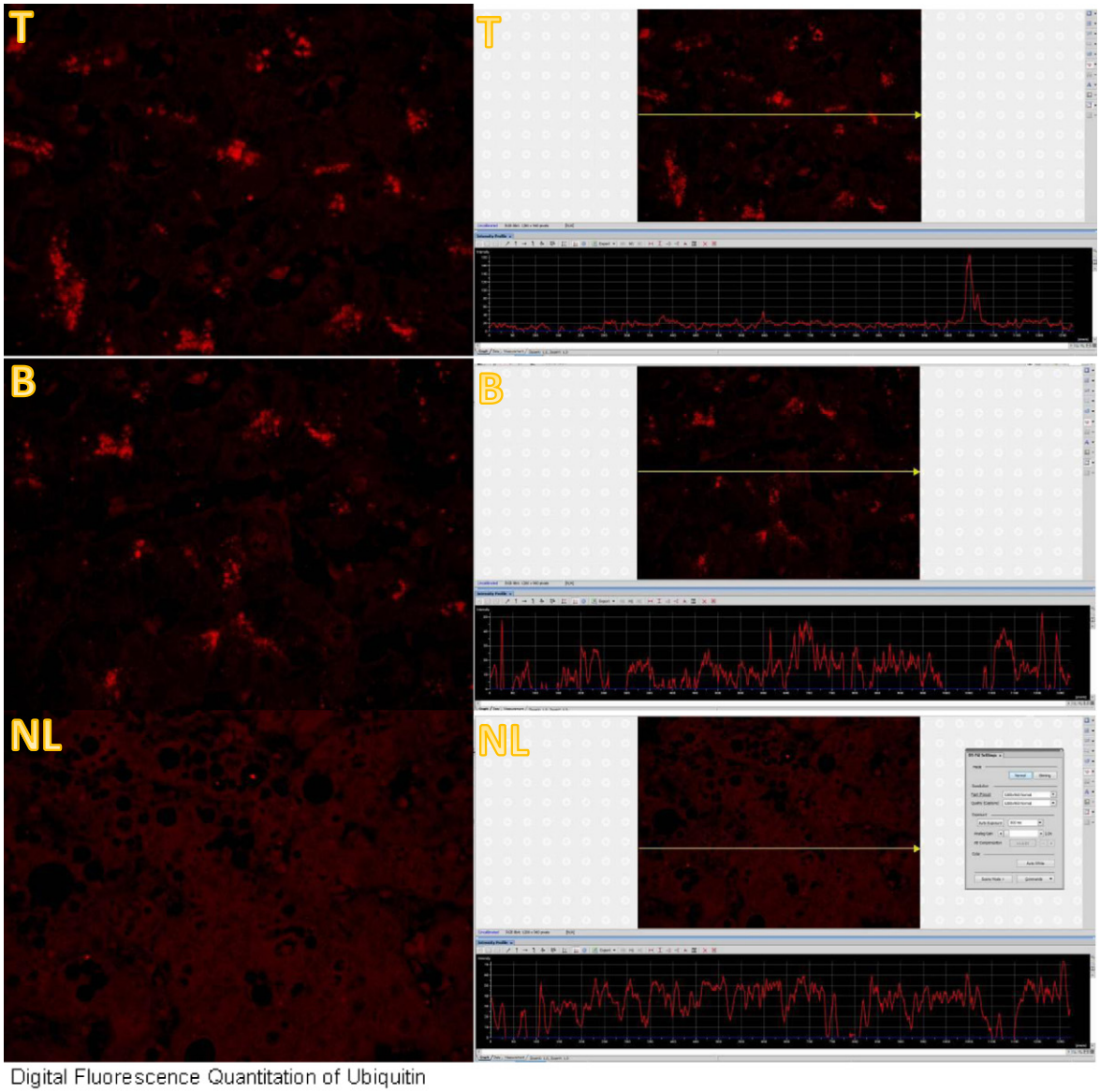


Fig. 7. T and B resections showed a decrease in ubiquitin. Fluorescence intensity profiling of the stained tissue array presented lower red intensity peaks than normal resections. The average intensities for T and B were found lower than control resections. The results are shown as Mean ± S.E.

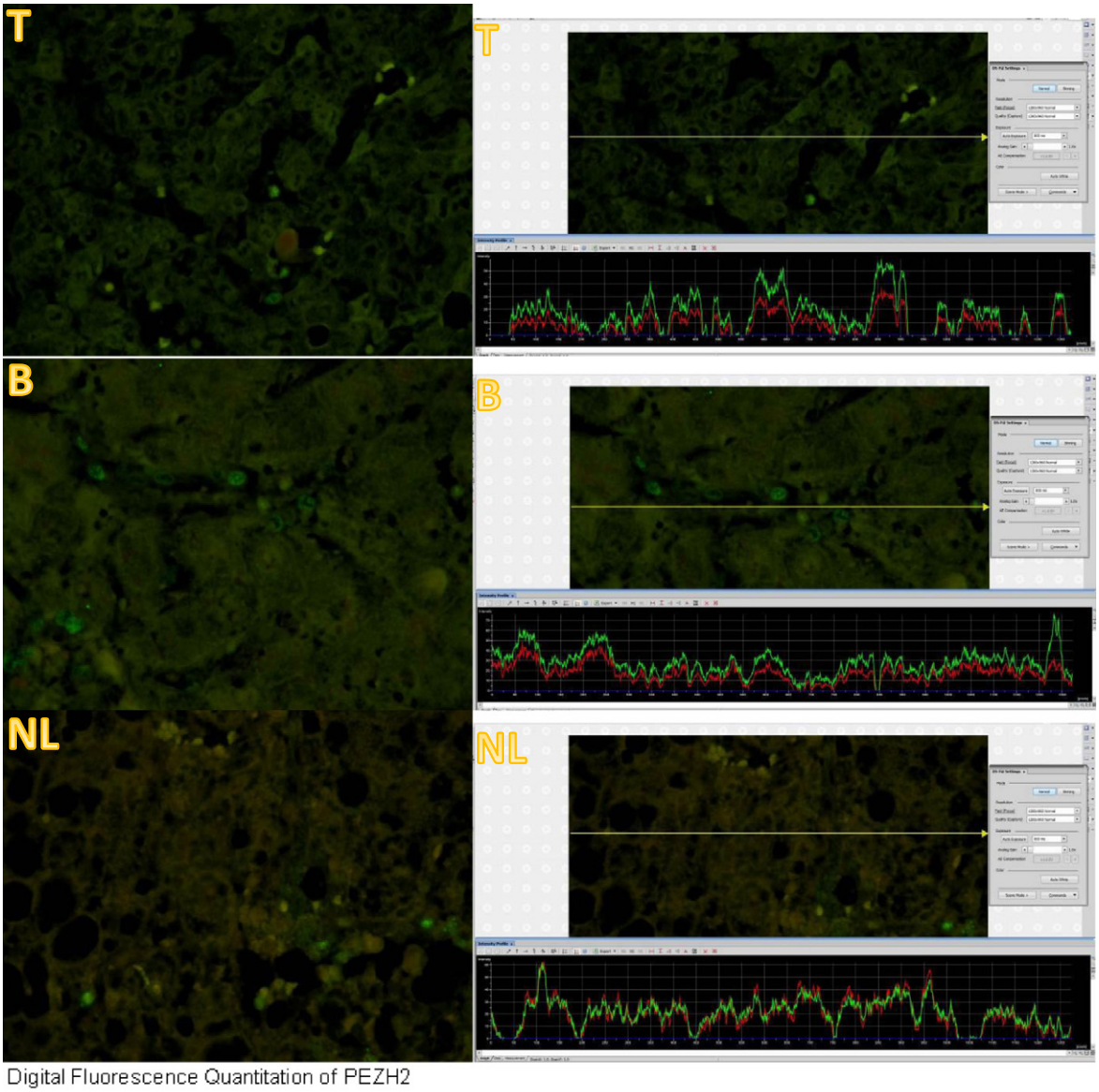


Fig. 8. T resections indicated a small decrease in pEZH2. Fluorescence intensity profiling indicated smaller peaks of fluorescence intensity; no significant change between B and NL was observed. The average fluorescence intensities indicate no significant difference between B and NL resections; the results, shown as Mean ± S.E., only indicate a small decrease in T resections.

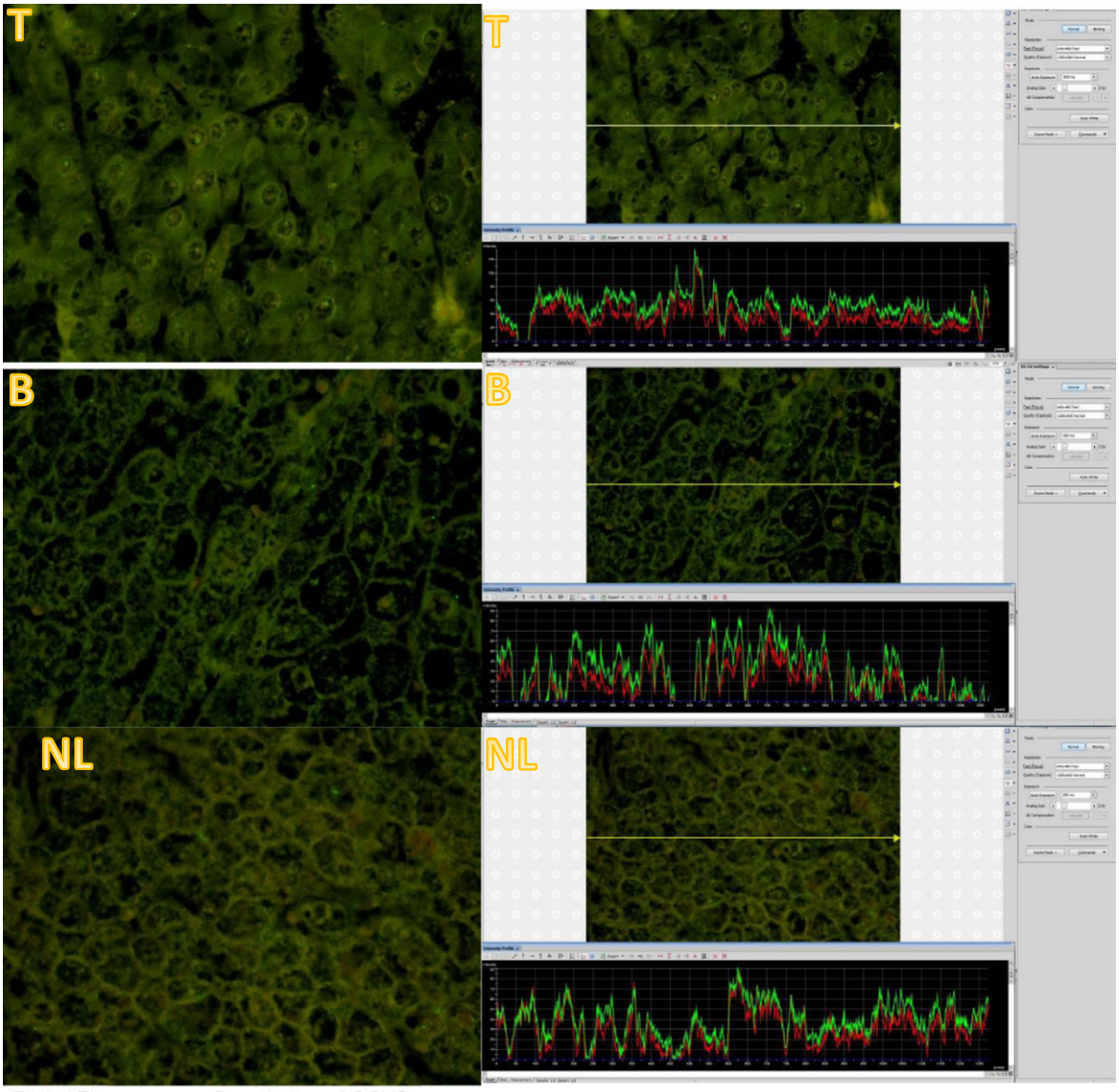


Fig. 9. SOX2 did not vary between the T, B, and NL resections. Fluorescence intensity profiling of T and B resections demonstrated no large change in fluorescence. The average intensities, shown as Mean ± S.E., indicate very little change between T, B, and NL resections.

and B liver tissues. Nanog levels are regulated by pEZH2 and H3K27me3 reversing the de-differentiation by Nanog. This is done by downregulating it to cause cellular differentiation (Villasante et al., 2011). PEZH2 directly regulates the epigenetic status of the Nanog promoter affecting the balance of Nanog expression in pluripotential stem cells and therefore, the equilibrium between self-renewal and differentiation (Villasante et al.,

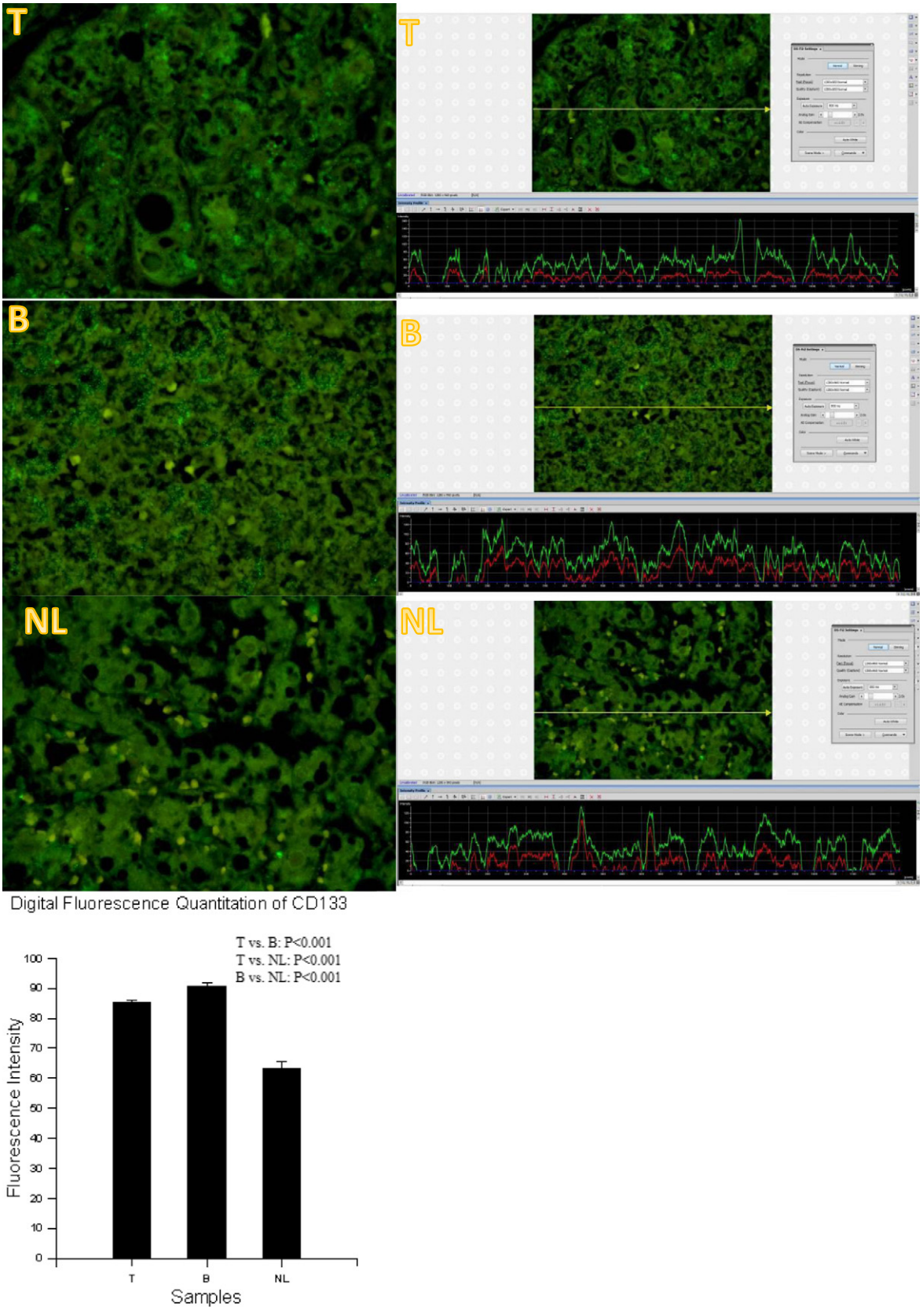


Fig. 10. CD133 was upregulated in B and T resections. Fluorescence intensity profiling revealed higher intensity peaks in B and T resections. The average intensities indicate a significant increase in B and T resections. Results shown as Mean ± S.E.

2011). Nanog levels are also increased in tumor-initiating stem-like cells (TISC) in response to TLR4 (Chen et al., 2013; Machida et al., 2012). TLR4 is upregulated in HCCs (Eiro et al., 2014).

The TISC expressed the highest intensity with the stem cell marker CD133. CD133 and CD49f are the markers used to identify TISC (Machida et al., 2012). CD49f was decreased in the HCC tissue compared

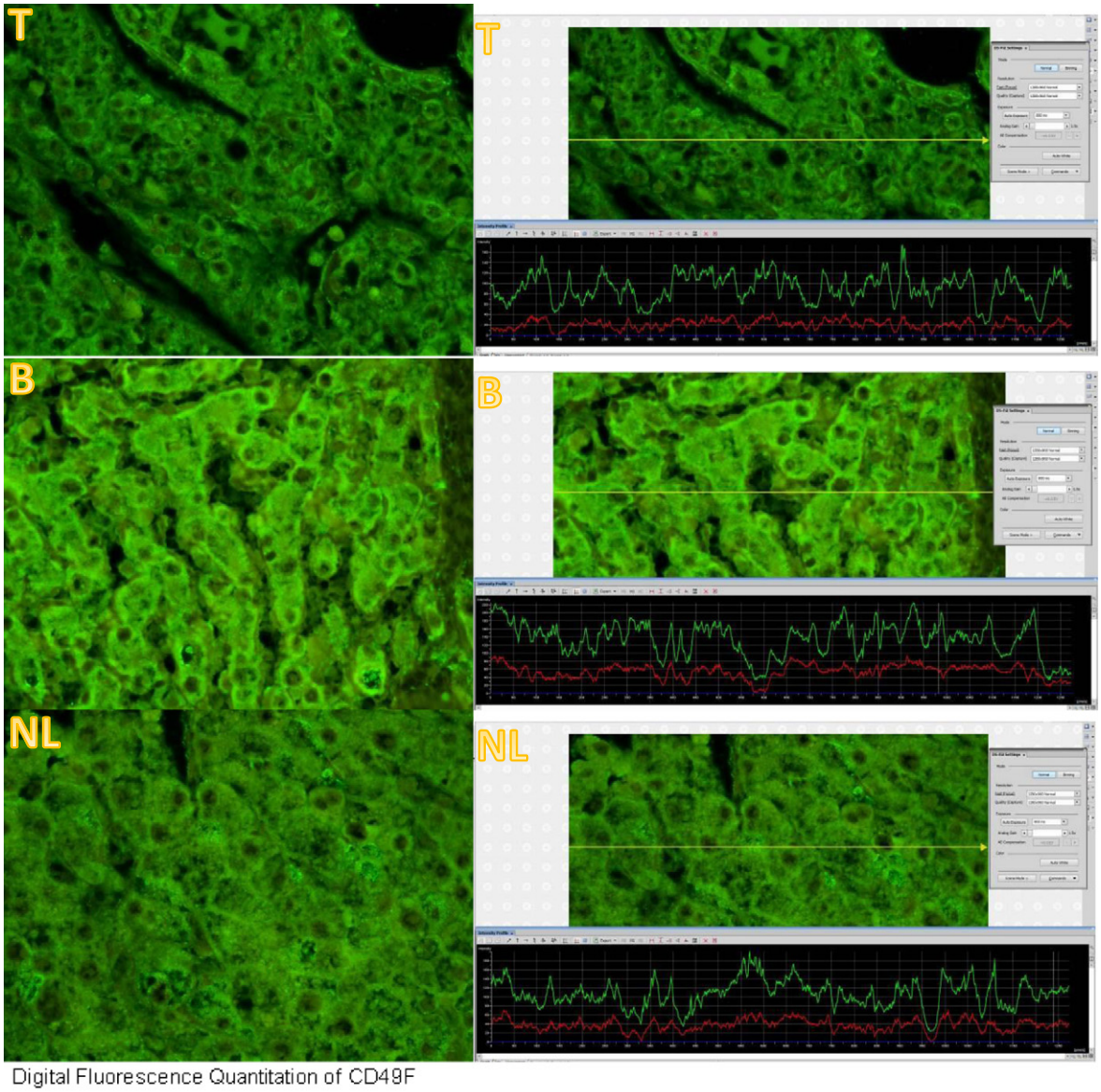


Fig. 11. CD49F decreased in T and B resections. Fluorescence intensity profiling indicates smaller peaks in T and B resections. The resulting average intensities, shown as Mean ± S.E., indicate a decrease in T resections and a slight decrease in B resections.

to the parent tissue and controls. CD133 has been used as the Tumor Initiating Cells' (TIC) marker in HCC (Mansour et al., 2014). Increase in circulating CD133 positive cells in patients with HCC correlated with age, TGFß, LN metastases and portal vein tumor thrombosis suggesting that the CD133 increased expression was driven by TGFß (Mansour et al., 2014). GPR87 overexpression upregulates CD133 expression,

which indicated that GPR87 promoted the growth, invasion and metastasis of CD133 cancer stem-like cells and increased tumor initiation in vivo (Yan et al., 2013).

5. Conclusion

The only stem cell marker that was upregulated in HCC was CD133. The stem cell markers Nanog, pEZH2, SOX2, OCT3/4 and CD49F showed a decrease. The results of FAT10 and ubiquitin are consistent with reduced protein quality control activity. The present study shows that the decrease in the tumor suppressor pEZH2 allows the increase in the expression of the stem cell marker, CD133, which increases causing hepatocyte de-differentiation, proliferation, and the formation of HCC.

Conflict of interest

There is no conflict of interest.

Acknowledgments

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