Fluorine-18 Fluorodeoxyglucose Uptake in Hepatocellular Carcinoma: Correlation with Glucose Transporters and p53 Expression

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Introduction: Hepatocellular Carcinoma (HCC) is one of most lethal cancers worldwide. The prognosis is very poor and therapeutic options are limited. The aim of this study was to determine the correlation of the [¹⁸F]FDG uptake profile of three HCC cell lines with p53 and glucose transporters (GLUTs) 1, 2, 3, 5 and 12 expression and with the glucose level present in the cell culture medium. **Methods:** Cell lines used are HepG2 (wp53), HuH7 (overexpress p53) and Hep3B2.1-7 (p53null). An immunocytochemical analysis was performed to evaluate p53 expression. Through uptake studies were analyzed the [¹⁸F]FDG uptake profiles of all cell lines under study. The expression of GLUTs were quantified by flow cytometry. The [¹⁸F]FDG uptake studies GLUTs expression analysis were performed on cells that grew in a high and low glucose medium in order to determine the effect of glucose concentration on GLUTs expression and on [¹⁸F]FDG uptake. **Results:** Immunocytochemical analysis confirmed the p53 expression profiles of all cell lines. It was found out that for all cell lines, [¹⁸F]FDG uptake is higher when cells grow in low glucose medium, however, the glucose level doesn't affect mostly the GLUTs expression. The Hep3B2.1-7 (p53null) is always the one that have higher [¹⁸F]FDG uptake. It was found that not always GLUT1 and GLUT3 are the most expressed by these cell lines. **Conclusions:** Our results shown that the p53 expression influences [¹⁸F]FDG uptake. This suggests that [¹⁸F]FDG may be used in HCC diagnosis, and may even provide some information about the genetic profile of the tumor. (J CLIN EXP HEPATOL 2015;5:183–189)

Hepatocellular Carcinoma (HCC) is one of most lethal cancers, with an increasing incidence in several regions of the world. Without specific treatment, the prognosis is very poor and survival is not prolonged. The most effective therapies are liver transplantation and surgical resection; however, only 15% of patients are candidates for these therapies. Therefore a wide range of patients are subjected to conventional chemotherapy and radiotherapy, in most cases without success.^{1–4} This happens because there are differences in the response to

these therapies due, at least in part, to the heterogeneity in expression of genes linked to chemoresistance and radioresistance, where the tumor suppressor gene p53 has a very important role.^{5,6}

Thus, it is important to find diagnostic tools to identify these genetic differences in order to develop more personalized therapies and increase the therapeutic success.

One of the most used approaches for cancer diagnosis is the Positron Emission Tomography (PET) using the radio-labeled glucose analog fluorine-18 fluorodeoxyglucose ([¹⁸F]FDG). The emergence of the central role of PET with [¹⁸F]FDG for the imaging evaluation of patients with cancer is undeniable. [¹⁸F]FDG PET has been used for diagnosis, initial staging, restaging, course, and monitoring of treatment response, surveillance, and prognosis in a variety of cancers.⁷

Although this imaging technique is not widely used for liver tumors, recent studies have shown that PET enables the identification of different uptake profiles in tumors of the same organ, namely HCC, but with different genetic profiles, which indicates that PET can be used in the diagnosis of liver cancers, leading the patients to a more personalized therapies.^{8–10}

Keywords: genetic profile, GLUTs, hepatocellular carcinoma, p53, [¹⁸F]FDG

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Abbreviations: HCC: hepatocellular carcinoma; GLUTs: glucose transporters; PET: Positron Emission Tomography; [¹⁸F]FDG: fluorine-18 fluorodeoxyglucose; CPM: counts per minute; HG: high glucose; LG: low glucose; PBS: phosphate buffer solution

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Increased [¹⁸F]FDG uptake based on enhanced glucose metabolism in cancer cells is a sensitive marker of tumor viability. [¹⁸F]FDG is introduced into tumor cells via glucose transporters (GLUTs) and converted to [¹⁸F]FDG-6 phosphate, which is not a substrate for subsequent enzymatic reactions.^{11,12}

GLUT1 and GLUT3 were originally identified as glucose transporters with primary role in the transport of [¹⁸F]FDG, since it is known that these transporters are over-expressed in tumor cells. However, more recently, GLUT5 and GLUT12 also appeared as transporters with an active role in this process, in particular in breast cancer cells.^{7,13,14} GLUT2 is a transporter fairly expressed in normal liver which may play a significant role in HCC.¹⁵ Furthermore, clinical studies have shown that [¹⁸F]FDG uptake can be also related with p53 protein expression in various tumor cell lines. Accordingly, some studies have shown that mutations in p53, or absence of expression of this protein are associated with high levels of [¹⁸F]FDG uptake.^{16,17}

Thus, the objective of this experimental work was to establish the [¹⁸F]FDG uptake profile of three HCC human cell lines and correlate them with the expression levels of p53, GLUT1, GLUT2, GLUT3, GLUT5 and GLUT12.

METHODS

Cell Culture

The human HCC cell lines used are HepG2, HuH7 and Hep3B2.1-7. The HepG2 and Hep3B2.1-7 were obtained from the American Tissue Cell Collection (ATCC), USA. The HuH7 cell line was obtained from the Japanese Collection of Research Bioresources (JCRB), Japan. According to the literature these cell lines differ in p53 expression, HepG2 cells express normal P53 (wp53), HuH7 and Hep3B2.1-7 express different mutated forms of p53 (mp53), thus, the HuH7 cell line overexpress p53 and Hep3B2.1-7 don't express this protein.¹⁸ Cells were propagated on adherent cultures in Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS) (Gibco 2010-09), 100 units/ml of penicillin and 100 µg/ml of streptomycin (Gibco 15140-122), pH 7.4 and incubated at 37 °C in 5% CO₂ atmosphere. We used two different formulation of DMEM medium with high glucose (HG) (25 mM) (Sigma D5648) and with low glucose (LG) (5 mM) (Gibco 31600-091), in order to clarify the influence of the glucose concentration on [¹⁸F]FDG uptake and in the expression of GLUTs.

p53 Expression Analysis

An immunocytochemical analysis was performed to evaluate the p53 expression. 10⁶ cells of each cell line under study were centrifuged at 500 g during 5 min. Subsequently, the pellets were fixed in formalin and embedded in paraffin to allow immunocytochemistry. Thus, the

analysis was performed using formalin-fixed, paraffin-embedded sections. Then it was performed the avidin-biotin peroxidase complex technique using monoclonal antibody anti-p53 (Dako M7001) at 1:50 dilution. The samples were observed under a light microscope—Nikon Eclipse 50i, and images were obtained using a Nikon-Digital Sight DS-Fi1 camera. The p53 was evaluated by percentage of expression.

[¹⁸F]FDG Uptake Studies

The uptake studies were performed according to a protocol already described.^{19,20} The cells were cultured in two different culture medium, HG and LG. The HG culture medium (with 25 mM of glucose) was the one who was designated by the supplier. The LG culture medium (with 5 mM of glucose) mimics the normal human glycemic. To perform cell studies, cells were washed with phosphate-buffered saline (PBS), then harvested with a solution of 0.25% trypsin/EDTA and finally resuspended in cell culture medium at 2 × 10⁶ cells/ml in 25 cm² flasks. The flasks were incubated at 37 °C in 95% O₂ and 5% CO₂. Subsequently, 0.925 MBq/mL of [¹⁸F]FDG was introduced in the cell culture medium. Duplicate samples of 200 µl were removed to microtubes containing chilled PBS for determination of tracer uptake at 1, 5, 15, 30, 45, 60, 90, 120, 150, 180, 210 and 240 min. During tracer uptake studies, for every sample taken, the cells were resuspended in order to ensure uniformity. Cell suspensions were then centrifuged at 5600 g for 60 s, followed by aspiration of the supernatant. Radioactivity of cell pellets and supernatants were measured separately with a dose calibrator (CAPINTEC CRC-15W) in counts per minute (CPM) in order to determine the [¹⁸F]FDG uptake percentage by the cells. The experiment was performed on three different days.

Cell Viability Analysis

Cell viability was assessed by trypan blue exclusion assay at the moment of the conclusion of experiments.^{19,20}

GLUTs Expression Analysis

The membrane and cytoplasmic expression of GLUTs 1, 2, 3, 5, 12 was analyzed by flow cytometry. To evaluate the membrane expression of the referred glucose transporters, 10⁶ cells were washed by centrifugation with PBS (300 g during 5 min). Cells were stained with 1 mg of monoclonal antibody anti-GLUT1-PE (PE, phycoerythrin) (R&D Systems MAB1349), or 1 mg of monoclonal antibody anti-GLUT2-PE (R&D Systems FAB1414P), or 1 mg of monoclonal antibody anti-GLUT3 (R&D Systems MAB1415), or 1 mg of monoclonal antibody anti-GLUT-5 (R&D Systems MAB1349) or 1 mg of monoclonal antibody anti-GLUT-12 (Santa Cruz Biotechnology sc-161659) during 15 min at room temperature and in the absence of light. Posteriorly,

the cells stained with monoclonal antibodies anti-GLUT1-PE and anti-GLUT2-PE were washed with PBS by centrifugation at 300 g during 5 min and resuspended in 400 mL of PBS. Stained cells with the antibodies anti-GLUT3, anti-GLUT5 and anti-GLUT12 were washed with PBS by centrifugation (300 g during 5 min) and labeled with 1 mg of a anti-mouse secondary antibody conjugated with PE (Santa Cruz Biotechnology sc-3818) for GLUTs 3 and 5 or 1 mg of anti-rabbit secondary antibody conjugated with PE (Santa Cruz Biotechnology sc-3755) for GLUT12, during 20 min at room temperature and in the absence of light. After incubation, cells were washed with PBS by centrifugation (300 g during 5 min) and resuspended in 400 mL of PBS. To evaluate the cytoplasmic expression of the studied glucose transporters, 10^6 cells were washed with PBS as above. Cells were resuspended in 100 μ L of Solution A (Fixative solution—Kit IntraCell (ImmunoStep)) and incubated during 15 min at room temperature in the absence of light. Afterwards, cells were resuspended in 100 μ L of Solution B (Permeabilitive solution—Kit IntraCell (ImmunoStep)) and stained with monoclonal antibodies anti-GLUT1-PE, anti-GLUT2-PE, anti-GLUT3, anti-GLUT5 and anti-GLUT12, as described above.

Statistical Analysis

Using the program OriginPro8, the uptake curves were fitted to an exponential model:

$$U(t) = A(1 - e^{-\frac{\ln(2)}{T}t})$$

where $U(t)$ is the uptake at t time, t is the time (minutes), A is the maximum uptake corresponding to steady state). This model allows to calculate the time required for uptake reached a value equal to $A/2$, parameter that we designate as T .

Statistical analysis was performed using IBM® SPSS® v. 20.0 (IBM Corporation, Armonk, New York, USA). Normality assessment for quantitative variables was executed with Shapiro-Wilk's test, in order to determine the use of parametric or nonparametric tests. For GLUT expression, comparisons between cytoplasmic and membrane expression, as well as comparisons between glucose levels, were executed using either Student's t -test (parametric), if the

distribution was normal, or Mann-Whitney's nonparametric test otherwise; for comparisons between cell lines, one-factor analysis of variance (ANOVA) test (parametric) was used, if a normal distribution and homogeneous variances were present, otherwise Kruskal-Wallis nonparametric test was used. Multiple comparisons were obtained with Bonferroni's correction.

In the uptake studies, comparisons of the parameters A and T between glucose conditions was performed using Student's t -test; for comparisons between cell lines, one-factor ANOVA test was used, with multiple comparisons performed using Bonferroni's correction.

A significant level of 5% was adopted for all comparisons.

RESULTS

p53 Expression

The Immunocytochemical analysis for HEPG2 cells registered a nuclear stain for p53 in about 30% (Figure 1a). Regarding the HuH7 cell line it was registered a nuclear stain for p53 in about 80% (Figure 1b). Finally the Hep3B2.1-7 cells had an immunostain for p53 (Figure 1c) in the nucleus.

[18 F]FDG Uptake Studies

Differences were observed in [18 F]FDG uptake studies, between the [18 F]FDG uptake by the three cell lines and between the two media formulation. For all cell lines, it was found that the [18 F]FDG uptake percentage is greater in LG medium than in HG medium (Figure 2). In both media formulations, the cell line that had higher [18 F]FDG uptake was Hep3B2.1-7 followed by HuH7 and finally HepG2 (Figure 2).

For HepG2 cell line it was observed that varying the glucose concentration of the cell culture medium the parameters A and T has higher values when the cells grow in a LG medium, differences being statistically significant ($P < 0.001$) between A values.

Regarding the HuH7 cell line, the values of A and T are also higher when the cells grow in LG medium, showing

Figure 1 p53 expression by HCC cell lines. Immunocytochemical analysis of p53 expression in HepG2 (a), HuH7 (b) and Hep3B2.1-7 (c) cell lines. Images were acquired under a magnification of 400X. HepG2 cell line express p53, HuH7 cell line overexpress this protein and Hep3B2.1-7 cells do not express p53.

Figure 2 [¹⁸F]FDG uptake profile of HCC cell lines. [¹⁸F]FDG uptake (%) by HepG2 cell line (a), HuH7 cell line (b) and Hep3B2.1-7 cell line (c). Cells grown in DMEM high glucose medium (25 mM) and in DMEM low glucose medium (5 mM). The figure represents the mean and standard deviation of four independent experiments.

differences statistically significant in both parameters ($P < 0.001$). Finally, similarly to what happens with the other cell lines, for the Hep3B2.1-7 cells, the A and T values are higher when the cells grow in LG medium, showing statistically significant differences only for the A parameter ($P < 0.001$).

Comparing the three cell lines, when cells grow on high glucose medium, the A and T values are higher in the Hep3B2.1-7 cell line followed by the HuH7 cell line and finally by the HepG2 cell line, with statistical significant differences, only in case of A parameter and between all cell lines ($P < 0.001$) (Table 1).

When the cells grow in LG medium, the A parameter is higher to the Hep3B2.1-7 cell line, followed by the HuH7 and lastly HepG2 cells in which the statistically significant differences are between HepG2 cells and HuH7 cells ($P < 0.001$) and between the cell lines HepG2 and Hep3B2.1-7 ($P < 0.001$) (Table 1). Regarding the T parameter, it is higher to the HuH7 cell line, followed by Hep3B2.1-7 and finally HepG2, with statistical significant differences between HuH7 and HepG2 cell lines (Table 2).

The trypan blue exclusion assay revealed that more than 95% of the cells were alive at the conclusion of all experiments.

GLUT's Quantification

Observing the Figure 3 it is noted that GLUT's cytoplasmic expression was always higher than the corresponding membrane transporters expression. These results always have differences with statistical significance ($P < 0.05$) except for GLUT3 in HuH7 cell line when grown in HG medium, and for GLUT1 in the same cell line, when cultured in LG medium (Figure 3 b and e).

In GLUTs expression (cytoplasmic or membrane) between cells cultured in LG medium and cells cultured in HG medium, there are differences for GLUT3 membrane expression in HepG2 cell line (Figure 3 a and d), GLUT1 and GLUT12 cytoplasmic expression in HuH7 cell line (Figure 3 b and e) and GLUT2, GLUT3 and GLUT12 cytoplasmic expression, and GLUT5 membrane expression in Hep3B2.1-7 cell line (Figure 3 c and f).

Observing the expression levels of each GLUT in the three cell lines under study, it was found that the GLUT1's membrane is more expressed in HuH7 cell line, followed by HepG2 and finally by the Hep3B2.1-7's cells ($P < 0.05$). Regarding the cytoplasmic expression of this transporter, this is more expressed in HepG2 cell line, followed by HuH7 and Hep3B2.1-7 ($P < 0.05$) (Figure 3).

Table 1 Maximum Uptake of [¹⁸F]FDG Uptake. The Table Represents the Average of A (% of Maximum Uptake, Steady State) for the Three Cell Lines Studied, in Both Cell Culture Media. r^2 Values Obtained was Always Greater than 0.94.

Cell line	Glucose	A (uptake %)	Standard error
HepG2	LG	3.37	0.37
	HG	1.14	0.01
HuH7	LG	16.60	0.75
	HG	1.76	0.08
Hep3B2.1-7	LG	18.16	1.64
	HG	3.47	0.28

Table 2 Time Required to Reach 50% of [¹⁸F]FDG Uptake. The Table Represents the Average of T (Time Required for Uptake is Equal to A/2, in Minutes) for the Three Cell Lines Studied, in Both Cell Culture Media. r^2 Values Obtained was Always Greater than 0.94.

Cell line	Glucose	T (minutes)	Standard error
HepG2	LG	13.62	2.73
	HG	0.15	0.31
HuH7	LG	54.25	8.43
	HG	5.02	1.95
Hep3B2.1-7	LG	25.96	8.38
	HG	22.51	6.35

Figure 3 Glucose transporters expression by HCC cell lines. Membrane and cytoplasm glucose transporters 1, 2, 3, 5 and 12 expression in HepG2 (a and d), HuH7 (b and e) and Hep3B2.1-7 (c and f) cell lines. Cells grown in high glucose medium (25 mM) (a, b, c) and in low glucose medium (5 mM) (d, e, f). The expression levels are expressed as mean intensity fluorescence (MIF). The figure represents the mean and standard deviation of four independent experiments.

Regarding the GLUT2 There were no statistically significant differences between the expression of this transporter (cytoplasmic or membrane) between the cell lines analyzed. This was observed when cells were cultured in culture HG and LG culture medium. However, there is a slight tendency for this carrier to have a more abundant expression by the cell line Hep3B2.1-7, both the cytoplasm and the membrane. (Figure 3).

In relation to GLUT3, in membrane, it is much expressed by Hep3B2.1-7 whereas the other two cell lines show a very similar expression and quite inferior to Hep3B2.1-7 ($P < 0.05$). In relation to cytoplasmic expression, HepG2 cells are those that have more expression than the transporter followed by Hep3B2.1-7 and HuH7 ($P < 0.05$) (Figure 3).

The GLUT5's membrane expression is quite similar for all cell lines studied. At cytoplasm, this transporter is more expressed by the Hep3B2.1-7's cell line than the other cell lines studied ($P < 0.05$) (Figure 3).

Finally, the cell line that more expresses GLUT12 in membrane is HuH7 followed by Hep3B2.1-7 and finally HepG2 ($P < 0.05$). While at cytoplasm, this transporter is more expressed by the Hep3B2.1-7's cells following by HuH7 and finally by HepG2 ($P < 0.05$) (Figure 3).

At membrane, the GLUTs more expressed by HepG2 cell line were 5 and 12, however, at cytoplasm GLUT1 is the more expressed transporter.

The HuH7 cells express more GLUT12 than any of the other GLUTs analyzed both at membrane and cytoplasm. In relation to the Hep3B2.1-7's cell line, at membrane, GLUT3 is the more expressed transporter, whereas at cytoplasm GLUT12 is the most pronounced transporter (Figure 3).

DISCUSSION

In this study, we characterized the cell lines studied regarding to p53 expression. We analyzed the [^{18}F]FDG uptake profiles of all HCC cell line in this study, and we also studied the expression of GLUT1, GLUT2, GLUT3, GLUT5 and GLUT12. Finally, we evaluated the influence of glucose level on [^{18}F]FDG uptake and GLUTs expression.

The immunocytochemical analysis confirmed the p53 expression profiles of the three HCC cells lines studied.¹⁸ Thus, it was found out that the HuH7 cell line is the one that has the greatest expression of this protein, followed by the HepG2 cells. It was confirmed the absence of p53 expression by the Hep3B2.1-7 cells.

For all cell lines studied, our results showed that the percentage of [^{18}F]FDG uptake is dependent on the glucose concentration in the cell culture medium. It was found that for all cell lines studied, the cells captured the [^{18}F]FDG uptake was higher when cells grown in LG cell culture medium in order to respond to their glycolytic requirements. When cells were propagated in HG cell culture medium with [^{18}F]FDG captured less, because they are not eager glucose. These results are consistent with what is observed in clinical practice, where plasma glucose concentrations affect the diagnostic value of PET using the [^{18}F]FDG.²¹ For this reason, in clinical practice when patients performing [^{18}F]FDG PET are fasted in order to not influence the sensitivity of the results. Thus, these data have a parallel with the results obtained in cell lines and here presented that prove the need of fasting for this diagnostic examination.²¹

It should also be noted that in both media formulations, the Hep3B2.1-7 cell line, which doesn't express p53,

is one that has the highest uptake percentage of [¹⁸F]FDG, and HepG2 cell line (which expresses the normal form of this protein) is always the one that has the lowest uptake percentage of this radiopharmaceutical. As mentioned previously, it appears that p53 expression can influence the [¹⁸F]FDG uptake.^{16,17} As described in the literature, also in our study the cells that do not express p53, which gives poor prognosis, are those that have the higher uptake of [¹⁸F]FDG.^{16,17,22}

There are already some explanations for the high rate of [¹⁸F]FDG uptake by the tumors that do not express p53, that express a minor p53 or express a non-functional form of this protein. Studies indicate that mutations in p53 decrease the suppressive effect of this protein in the genes that encode the GLUT1 and GLUT4.^{23,24} Moreover, the loss of the TIGAR (TP53 - induced glycolysis and apoptosis regulator) expression in tumors where p53 is not functional may also explain the high rate of [¹⁸F]FDG uptake.²⁵ Another study suggests that p53 suppression is associated with changes in glucose metabolism detected by PET.²⁶

Regarding the glucose transporters expression, it appears that there are differences between the cell lines studied. Interestingly, almost never occur significant differences in GLUTs expression (either membrane or cytoplasmic) between cells that grown in low glucose medium and cells grown in high glucose medium, indicating that the glucose level present in the cell culture medium, in most cases does not influence the GLUTs expression. These results are in agreement with the results obtained in a further study in which was used another HCC cell line.²⁷

It is noted that the glucose transporter more expressed at membrane are not always the most expressed at cytoplasm. In fact, some GLUTs have a low membrane expression with a remarkable cytoplasm expression (e.g. GLUT1 and GLUT3 expression in HepG2 cell line) which may indicate that they are highly expressed in the cytoplasm. Others have shown that GLUTs expressed at cytoplasm may mediate the glucose transport between cell membrane and endoplasmic reticulum, and this is one possible explanation for our results.²⁸

In relation to the GLUTs membrane expression, GLUT3 is one that is more expressed by the Hep3B2.1-7 cell line, which can be correlated with the fact that this cell line is that which has the highest [¹⁸F]FDG uptake percentage. The GLUT12 expression levels are very expressive in all cell lines, which is in agreement with the data presented by other studies indicating that this transporter has an active role in [¹⁸F]FDG uptake in other tumors.⁷ So it seems that also in HCC the GLUT12 may have a fairly active role in the transport of this radiopharmaceutical. Although the GLUT2 was described as the glucose transporter that is more expressed in normal hepatocytes,¹⁵ our study indicates that this transporter doesn't have major influence on [¹⁸F]FDG uptake in HCC cell lines studied, considering

that in these cell lines, among the GLUTs studied, the GLUT2 expression levels are little relevant.

Thus, these results indicate that, unlike that is described in the vast majority of the literature the glucose transporters 1 and 3 cannot be the only responsible for [¹⁸F]FDG uptake by cancer cells, and more specifically by HCC cells. These data lead us to conclude that, unlike data from previous studies the [¹⁸F]FDG can be used to HCC diagnosis, once may even provide some information about the genetic profile of the tumor, which can assist in the therapy choice.

CONCLUSIONS

In this study, it follows that the [¹⁸F]FDG uptake profile, in the same cell line, is different according to the availability of glucose in the cell culture medium. It was observed that not always the GLUT1 and GLUT3 are the primarily responsible for the [¹⁸F]FDG uptake by HCC cell lines under study. On the other hand, the GLUT12 membrane expression levels were quite significant in all cell lines studied, indicating that this transporter may have an active role in [¹⁸F]FDG uptake in HCC. The cell culture medium glucose level influences the [¹⁸F]FDG uptake. The results also indicate that the absence of expression of p53 or the non-functional expression of this protein leads to higher levels of [¹⁸F]FDG uptake in HCC, which is indicative of poor prognosis. Thus, the [¹⁸F]FDG uptake profile seems related to the genetic profile of each cell line, namely with p53 expression and studies with [¹⁸F]FDG in HCC may provide some information about tumoral genetic characteristics in HCC and may assist in the choice of the therapy.

CONFLICTS OF INTEREST

All authors have none to declare.

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